The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 53 MAY, 1959 NO. 5

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

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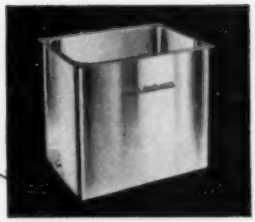

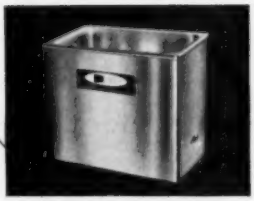

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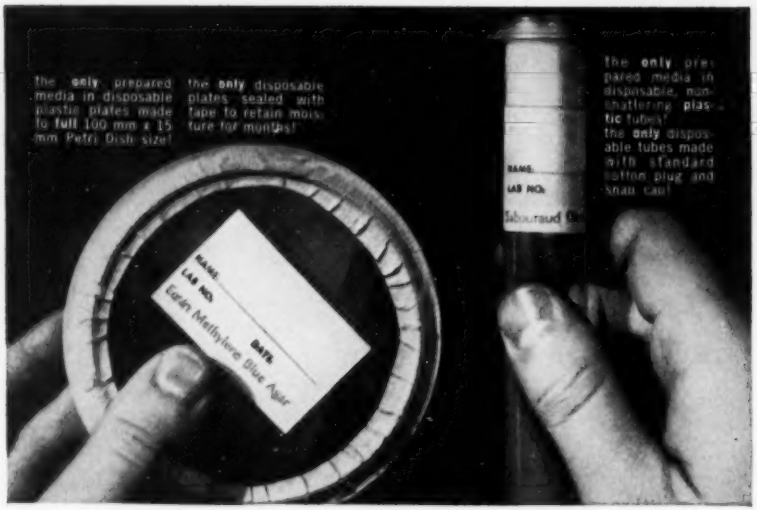
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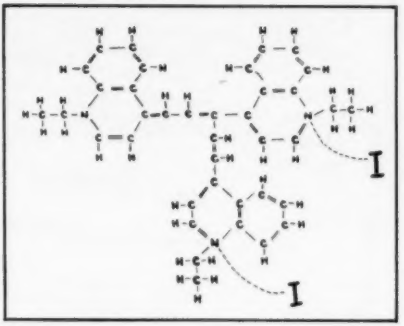

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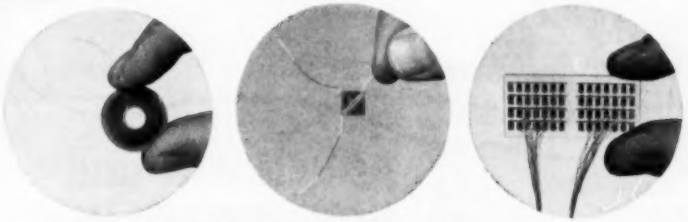

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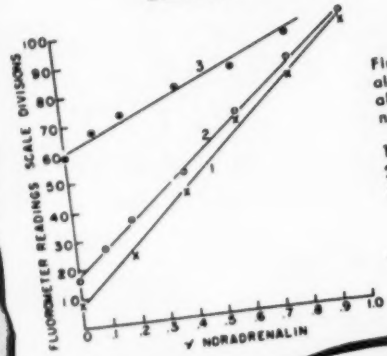

Fluorescence vs. amount of noradrenalin with varying amounts of adrenalin present, obtained according to noradrenalin method

1. Adrenalin, 0 γ; water reading, 6
2. Adrenalin, 1 γ; water reading, 6
3. Adrenalin, 5 γ; water reading, 6

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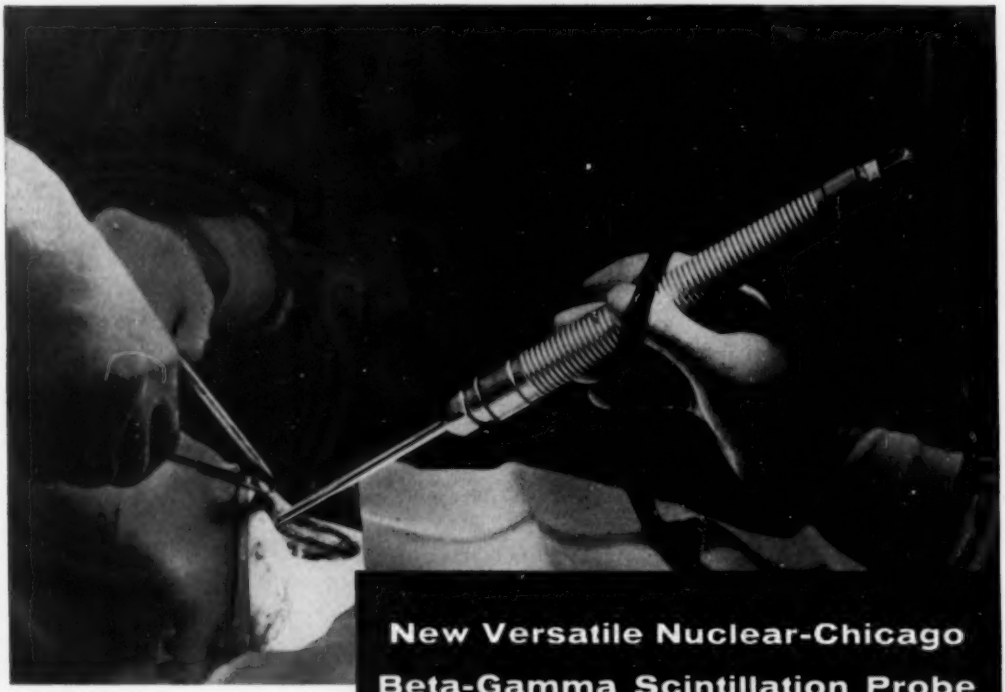

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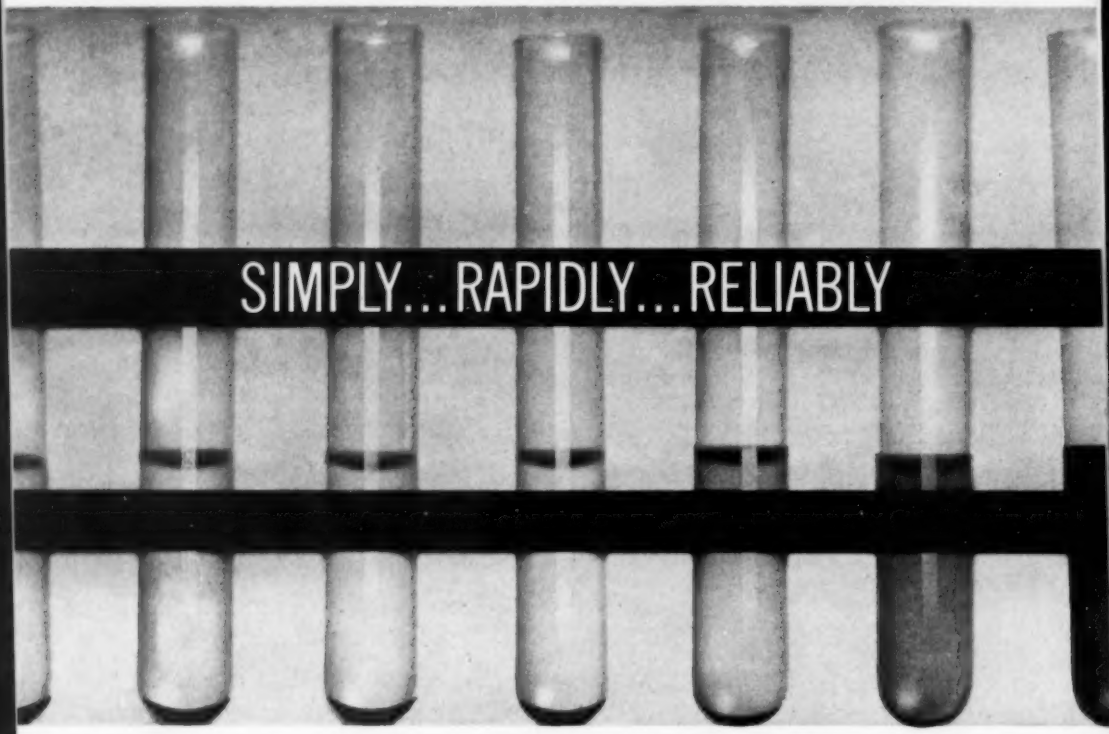

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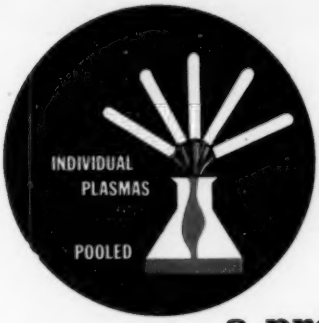

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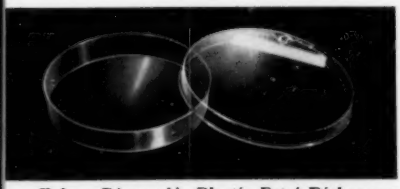

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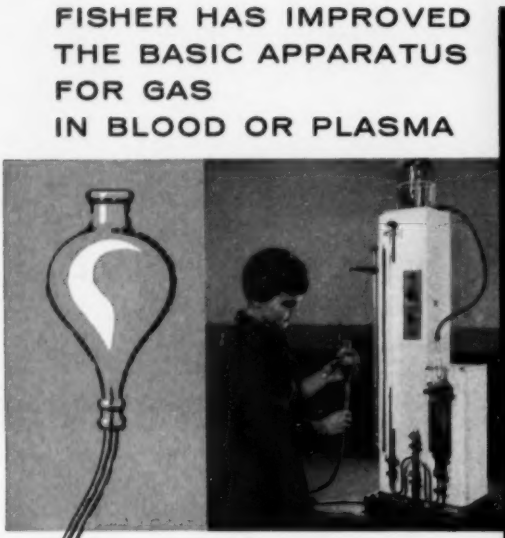

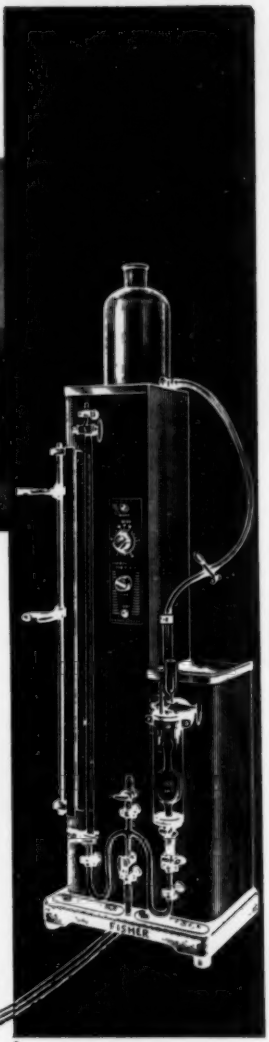

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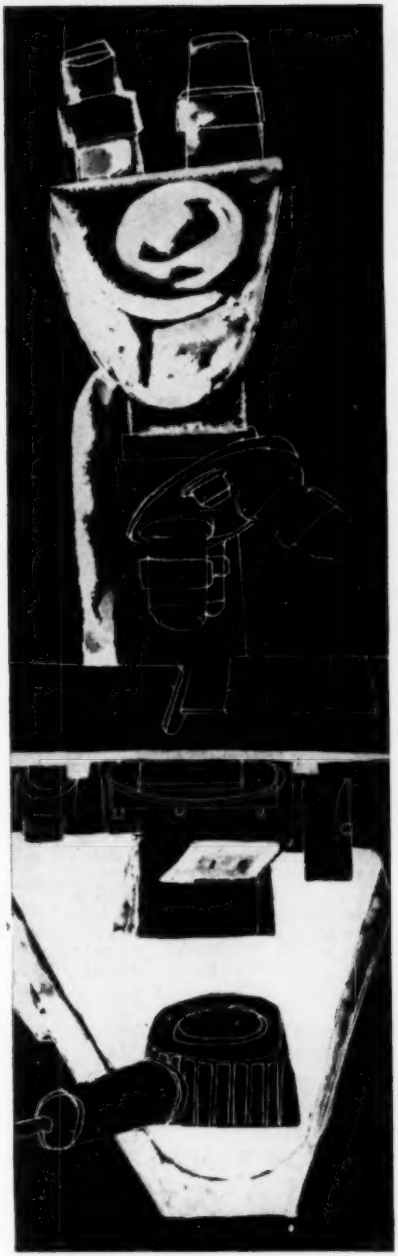

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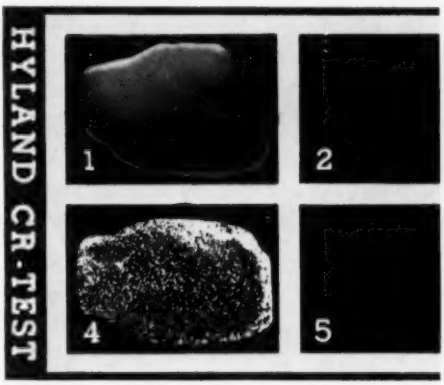

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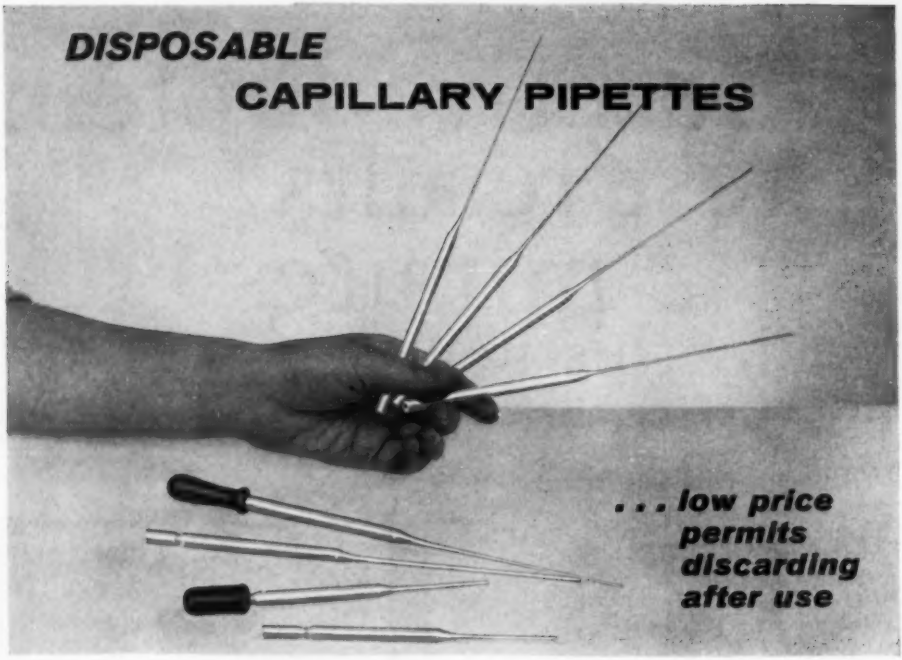

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VOL. 53 MAY, 1959 NO. 5

CLINICAL AND EXPERIMENTAL

THE PERSISTENCE IN THE BLOOD OF THE RADIOACTIVE LABEL OF ALBUMINS, GAMMA GLOBULINS, AND GLOBULINS OF INTERMEDIATE MOBILITY

VII. UPTAKE AND DIE-AWAY PLOTS OF S^{35} INTO SERUM ALBUMINS AND GAMMA GLOBULINS AFTER ORAL ADMINISTRATION OF LABELED METHIONINE IN IDIOPATHIC HYPOGAMMAGLOBULINEMIA AND MULTIPLE MYELOMA

DAVID BRONSKY, M.D., ALVIN DUBIN, M.S., AND DANIEL S. KUSHNER, M.D.
CHICAGO, ILL.

THE purpose of this paper is to present the uptake and die-away plots of S^{35}-labeled methionine into the serum proteins of subjects presenting the extremes of serum globulin concentrations found in idiopathic hypogammaglobulinemia and in multiple myeloma. This is part of a continuing study designed to seek valid reproducible means of estimating protein synthesis rates. Previous studies have demonstrated that the die-away plots for the S^{35} amino acid, because of reutilization of the label, lack consistency and that biologic turnover times calculated from them are unduly prolonged.[1, 2] We have, therefore, abandoned the die-away plot as a clinical approach to this problem. Those presented in this paper are the last we expect to do and are interpreted solely in qualitative terms.

Recent studies of the uptake plots for S^{35} methionine during the first 24 hours after oral administration, when presumably reutilization of the label is at a minimum, have shown promise of offering a reliable, although crude,

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These studies have been supported by Grant H-1511 from the National Heart Institute of the United States Public Health Service.

Received for publication Aug. 26, 1958.

quantitative method for estimating endogenous protein synthesis rates.[3] The present study is a companion to a previous report of protein synthesis rates in subjects with varying degrees of hypoalbuminemia.[3]

METHODS

Selection of Patients.—The subjects studied were 3 patients with idiopathic hypogammaglobulinemia and 4 with multiple myeloma. Simultaneously, 3 nutritionally normal male inhabitants of the Neurological Service (Huntington's chorea, cerebral palsy, and traumatic spinal cord paralysis) were studied as controls.

The patients with idiopathic hypogammaglobulinemia were white men, 36, 42, and 53 years old, respectively. Each presented a history of repeated infections, usually bacterial in type and pulmonary in location. Chronic sinusitis was present in two patients. This was associated with chronic bronchiectasis and clubbing of the fingers in one, and multiple, bacteriologically sterile, ulcerations of a leg in the other. This last patient also presented a persistent leukopenia with, however, adequate response to bacterial infection. All 3 patients had splenomegaly. Further studies demonstrated very low to absent isohemagglutinin and antistreptolysin titers and depressed zinc sulfate turbidity levels. Bone marrow aspirations and lymph node biopsies in all 3 patients demonstrated a paucity of plasma cells.

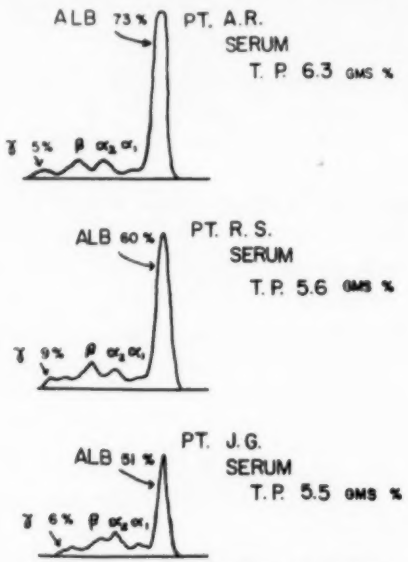

Fig. 1.—Serum protein paper electrophoretic diagrams in 3 patients with idiopathic hypogammaglobulinemia.

Diagrams of the serum protein paper electrophoretic patterns of these patients are presented in Fig. 1. Levels of the serum protein fractions are given in Table I. On free electrophoresis (Tiselius), substantially lower gamma globulin levels of 0.17, 0.39, and 0.10 Gm. per 100 ml. were found, respectively, for Patients A. R., R. S., and J. G.

The 4 male patients with multiple myeloma were selected to provide a cross section of the protein abnormalities found in this disease. The diagnosis was established by the usual clinical, biochemical, and hematologic methods, including bone marrow aspiration.*

*We are indebted to Dr. Stephen O. Schwartz and the Hematological Department of the Cook County Hospital for bone marrow studies.

Diagrams of serum protein electrophoretic patterns of these 4 patients and urine proteins of one patient are shown in Fig. 2. Levels of the protein fractions are presented in Table I. Two patients (J. F. and G. A.) had rapidly moving abnormal serum protein peaks which could be cleanly separated from small amounts of electrophoretically normal appearing gamma globulin. This allowed a simultaneous study of hypogammaglobulinemia and hyperglobulinemia in the same individual. Patient J. T. presented a slow moving gamma globulin peak. In Patient J. S., the protein abnormality consisted of the formation of large amounts of Bence Jones protein (bottom graph, Fig. 2). His serum protein partition was normal. None of the patients had a history of repeated infections. Upon autopsy, however, Patient J. F. presented extensive bronchopneumonia.

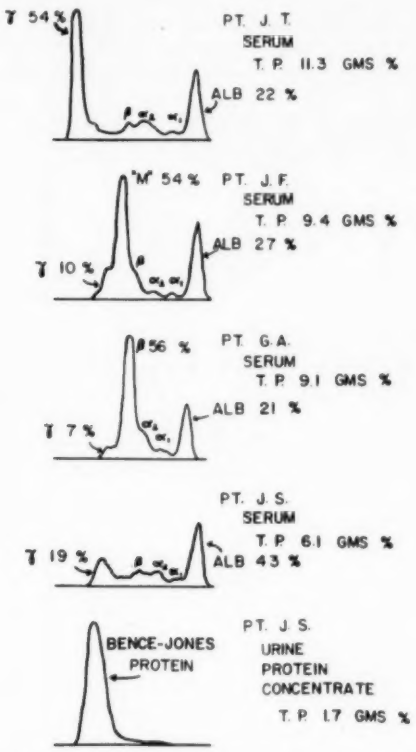

Fig. 2.—Serum and urine protein paper electrophoretic diagrams in 4 patients with multiple myeloma. For further description, see text.

Administration of Label and Radioactivity Measurements.—All subjects received oral S^{35} methionine, 0.50 mc. Supplemental oral protein hydrolysates, 2.5 Gm., were given at the time of labeling. Serial sera were drawn 2, 4, 8, and 24 hours after labeling, and then at frequent intervals from 14 to 38 days. Methods of fractionation, extraction, measurements of component concentrations and radioactivity have been described previously.[4]

To circumvent errors due to layering distortion of thin films when determining the radioactivity of small amounts of gamma globulin, duplicate separations of this fraction

TABLE I

DIAGNOSTIC GROUPS	PATIENT	SERUM LEVELS (GM./100 ML.) TOTAL PROTEIN	ALBUMINS	GAMMA GLOBULINS	OTHER	CORRECTED RADIOACTIVITY MAXIMA (C.P.M.) TOTAL PROTEIN	ALBUMINS	GAMMA GLOBULINS	OTHER
Idiopathic hypogammaglobulinemia	A. R. (1)	6.3	3.8	0.43		2,730	1,038	123	
	A. R. (2)	6.0	3.9	0.36		2,400	766	32*	
	J. G.	5.5	3.1	0.41		2,760	844	194	
								122*	
	R. S.	5.6	3.3	0.52		2,160	600	79	
								32*	
Multiple myeloma	J. F.	9.4	2.1	0.30	5.3†	2,640	632	115	1,395‡
	G. A.	9.1	1.3	0.59	6.1§	2,740	303	232	1,840‡
	J. T.	11.3	1.8		7.8‖	3,030	468		3,504‡
	J. S. (Serum)	6.1	3.0	1.31		2,978	1,312	618	
	J. S. (Urine)				1.7¶				2,081**
Nutritionally normal	J. M.	6.0	3.5	1.25		3,425	1,494	451	
	A. Ro.	7.3	3.2	1.30		3,045	896	375	
	J. J.	7.0	3.7	1.36		4,072	1,014	410	

*Duplicate study with added unlabeled bovine albumin to reduce layering film distortion.
†"M" globulin.
‡Expected normal gamma globulin levels used as correction factor (see text).
§Beta globulin.
‖Abnormal gamma globulin inseparable from normal gamma globulin.
¶Bence Jones protein both by thermal reactivity and electrophoretic characteristics.
**No correction factor applicable.

were performed in patients with idiopathic hypogammaglobulinemia and sufficient unlabeled bovine albumin was added to eliminate the need for applying a self-absorption correction factor based on thickness of film.[4]

The uptake plots were charted on regular graph paper and the die-away plots on semilogarithmic graph paper as corrected counts per minute against time in hours. These values by the corrections used are proportional to the specific radioactivity of the fractions measured in terms of counts per minute per gram of protein as corrected to a standard oral dose of 0.50 mc. In Table I, the eighth hour peak uptakes are further corrected by a ratio of the observed serum level to the expected normal level. By this means, a crude quantitation of the protein synthesis rate is obtained.[3]*

Chromatographic studies of S^{35} methionine recently obtained from Abbott Laboratories, Chicago, Illinois, have revealed an appreciable degree of contamination with S^{35} cystine. These plots, therefore, should be interpreted as primarily indicating S^{35} amino acid uptakes rather than methionine uptakes specifically.

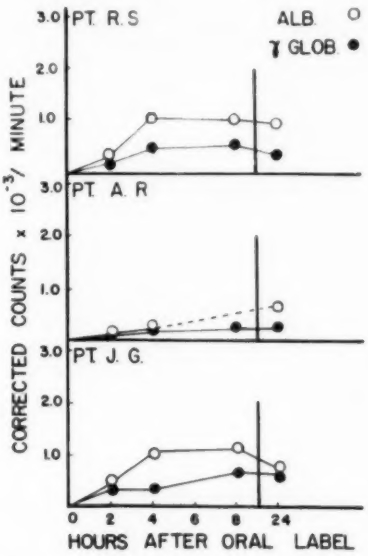

Fig. 3.—Uptake plots after oral S^{35} methionine in 3 patients with idiopathic hypogammaglobulinemia. These plots are corrected to a standard oral dosage and for radioactive decay only.

RESULTS

Idiopathic Hypogammaglobulinemia; Uptake Plots (Fig. 3).—Following oral administration of the S^{35} label, an appreciable uptake is present at 2 hours. Between the fourth and eighth hour, there is a plateau with a slight peak at the eighth hour. An early fall-off is apparent at 24 hours in both the albumin and globulin fraction. These plots follow the same general form previously described in normal and hypoalbuminemic subjects.[3] In each subject, the apparent incorporation of S^{35} into albumins is greater than into the

*Recently, Ulstrom and associates have independently arrived at the same correction factor in studying dysproteinemias of infants.[28]

corresponding gamma globulin fraction even when plots are not subjected to a correction based on an estimate of the circulating pool into which the newly synthesized protein is delivered.

The corrected maxima (see methods) for the eighth hour peak are presented in Table I. The synthesis rate for gamma globulin is markedly depressed. This inability to form gamma globulin is even more readily apparent when the error due to layering effect is eliminated (Table I).

The synthesis rate for albumins compares favorably with those for nutritionally normal subjects. The apparent depression in the synthesis rate for total proteins can be attributed to the decrement in gamma globulin formation (Table I).

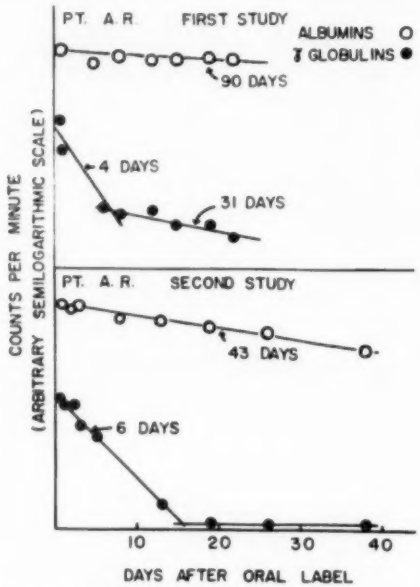

Fig. 4.—Duplicate studies of the die-away plots after oral S^{35} methionine in one patient with idiopathic hypogammaglobulinemia. In the bottom graph, unlabeled albumin was added to gamma globulin specimens to eliminate error due to layering effect (see Methods).

Idiopathic Hypogammaglobulinemia; Die-Away Plots.—The straight line exponential die-away plots for albumins conforms to the general form previously described.[5] The variations in apparent biologic half-life observed in duplicate studies on Patient A. R. (Fig. 4) again demonstrate our reasons for abandoning this method of study.

The steepness of the die-away plots for gamma globulin in Patients A. R. (Fig. 4) and R. S. (top, Fig. 5) contrasts strikingly to the gradual decline of the respective albumin plots. They differ also from gamma globulin die-away plots obtained in previous studies,[2, 5] for instead of a gradual flattening out 3 to 5 days after label administration, the slope continues its abrupt

descent for 15 to 18 days. The terminal horizontal portion of the plots were obtained when the amount of residual radioactivity was negligible. This finding suggests that, in these two patients, there is either an abnormally rapid degradation rate or failure of reutilization of the small amount of gamma globulin formed. In Patient J. G. (bottom, Fig. 5), however, the contrast between the albumin and gamma globulin plot is much less striking.

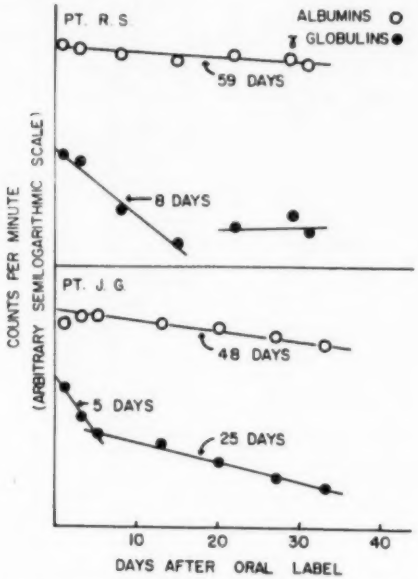

Fig. 5.—Die-away plots after oral S^{35} methionine in two patients with idiopathic hypogammaglobulinemia.

The apparent biologic turnover time for normal pooled Red Cross serum gamma globulin* was determined in Patient R. S. A total of 55 Gm. was administered intramuscularly in 10 days. The serum gamma globulin level rose from 0.40 to 0.67 Gm. per 100 ml. Subsequent study of the die-away plot using turbidimetric determination of gamma globulin demonstrated an apparent turnover time of 70 days. This is not only a much longer rate than that obtained in the same patient with endogenous S^{35} label, but the rate also exceeds the usual values of 25 to 36 days reported for other patients with idiopathic hypogammaglobulinemia after administration of similar amounts of gamma globulin.[6-8] This seemingly undue prolongation of the turnover time can perhaps be ascribed to experimental error introduced by failure to obtain a greater initial elevation of the gamma globulin level. This, in turn, can be attributed to the patient's large size (100 kg.).

*Generously supplied by Dr. Samuel T. Gibson of the American National Red Cross.

TABLE II. ELECTROPHORETIC PARTITION OF ABNORMAL AND NORMAL SERUM PROTEINS IN MULTIPLE MYELOMA AND IN NORMAL AGED ADULTS*

DIAGNOSTIC GROUP	NO. OF PATIENTS	MEASURED IN GRAMS PER 100 ML.						
		TOTAL PROTEIN	ALBUMIN	ALPHA-1 GLOBULIN	ALPHA-2 GLOBULIN	BETA GLOBULIN	GAMMA GLOBULIN	OTHER
Multiple myeloma with abnormal protein	37	10.61 ± 2.40	2.02 ± 0.73	0.35 ± 0.20	0.60 ± 0.30†	0.72 ± 0.18‡	0.72 ± 0.30§	6.20 ± 2.80*
Multiple myeloma with normal protein	9	6.50 ± 0.92	3.63 ± 0.87	0.33 ± 0.17	0.76 ± 0.45	0.79 ± 0.22	0.99 ± 0.49	
Normal aged adults‖ (70 to 94 years)	20	6.65 ± 0.33	3.45 ± 0.30	0.28 ± 0.04	0.71 ± 0.09	0.82 ± 0.10	1.31 ± 0.25	

*To construct this table, the abnormal serum globulin peaks were separated from the electrophoretically normal protein fractions. The mean and standard deviation of the abnormal fractions is presented under "other" and includes 23 patients with a gamma globulin abnormality, 12 with a beta globulin abnormality, and 2 in whom the abnormal globulin occupied both the beta and alpha-2 globulin position.

†Based on 35 patients.

‡Based on 26 patients.

§Based on 14 patients.

‖Aged adults are used for comparison because the majority of patients with multiple myeloma were over 50 years old.[9]

Multiple Myeloma; Uptake Plots (Fig. 6).—The form of the uptake plots again resembles those previously described. In each instance, the incorporation of the S^{35} label into the albumins appears greater than into serum globulin fractions. The uptake plots for Patient J. S. (bottom, Fig. 6), however, demonstrate that the apparent rate of incorporation of the label into Bence Jones protein exceeds that of either serum fraction.

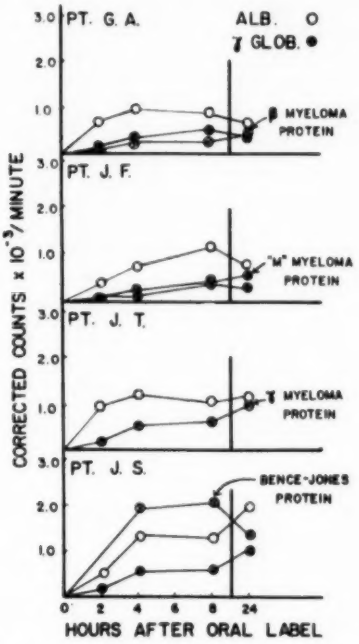

Fig. 6.—Uptake plots after oral S^{35} methionine in 4 patients with multiple myeloma, corrected as in Fig. 3.

The corrected 8-hour maxima (Table I) reveal a markedly rapid apparent synthesis rate for all abnormal proteins studied. In the two patients with electrophoretically demonstrable hypogammaglobulinemia (G. A. and J. F.), the apparent synthesis rates for this fraction are depressed to a level comparable with that found in idiopathic hypogammaglobulinemia. In all patients presenting an abnormal serum protein, there is also marked depression of the albumin apparent synthesis rates. However, in Patient J. S., whose only protein abnormality consisted of the formation of Bence Jones protein, the albumin and gamma globulin synthesis rates are normal. In spite of the formation of large amounts of abnormal globulins, the apparent total protein synthesis rate is, in general, somewhat less than that observed for normal individuals. This may be due to concurrent depression of the synthesis rate of the other serum protein fractions.

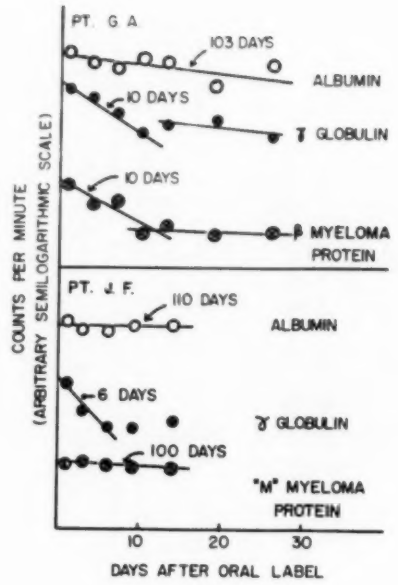

Fig. 7.

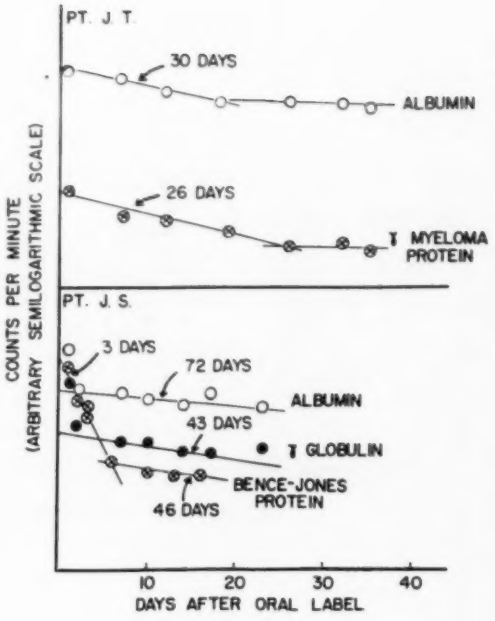

Fig. 8.

Fig. 7.—Die-away plots after oral S^{35} methionine in two patients with myeloma whose abnormal serum globulin could be clearly separated from a normal electrophoretic gamma globulin fraction.

Fig. 8.—Die-away plots after oral S^{35} methionine in two patients with multiple myeloma.

Table II offers additional information suggesting that the formation of an abnormal serum globulin fraction in patients with multiple myeloma is accompanied by a depressed level of other electrophoretic protein fractions.

Multiple Myeloma; Die-Away Plots.—The form of the die-away slopes for the abnormal myeloma protein and the normal gamma globulin fractions of Patient G. A. (top, Fig. 7) roughly parallel each other as well as the albumin fraction. In Patient J. F. (bottom, Fig. 7) the die-away slopes for albumin and "M" myeloma protein also parallel each other, but there is an apparent rapid decline of the normal gamma globulin fraction. This patient died 14 days after labeling and the validity of this observation is questionable. The remaining two patients (Fig. 8) demonstrate the same rough parallelism for the die-away slopes of the albumin and gamma myeloma protein (Patient J. T.) and normal gamma globulin (Patient J. S.).

The apparent turnover time of Bence Jones protein (bottom, Fig. 8) was extremely rapid as compared to the turnover of the corresponding normal serum gamma globulins. The secondary slope paralleling the serum gamma globulins apparently represents a period where the S^{35} label has become available for recycling from a free metabolic pool of amino acids.

DISCUSSION

The protein defect of idiopathic hypogammaglobulinemia has been attributed to an inability of these patients to synthesize adequate amounts of gamma globulin.[6-8] Experimental evidence for this consists of the observation of normal biologic turnover rates for pooled and I^{131}-labeled gamma globulin in individuals with very low gamma globulin serum levels. Additional evidence is provided by the paucity of plasma cells in the bone marrow and lymph nodes of these patients, for these cells are thought to be the source of the circulating gamma globulins.[7] The present study presents additional evidence that these patients are unable to adequately synthesize gamma globulin. Furthermore, the data in two patients indicate an abnormally rapid turnover rate of the small amounts of gamma globulin formed. It remains to be demonstrated whether the circulating gamma globulins are abnormal proteins or a normal fraction with a rapid turnover rate, for the apparent turnover time of normal gamma globulins is, in fact, a mean of the different turnover times of numerous specific globulin fractions.

The apparent biologic half-life of exogenous gamma globulin in patients with idiopathic hypogammaglobulinemia has been reported as approximately 25 to 36 days.[6-8] In the present study, the apparent biologic half-life for endogenously labeled gamma globulins in two patients with this syndrome was 6 to 8 days. This difference is striking, for all our previous experience has demonstrated undue prolongation of apparent turnover times when using the S^{35} label. The discrepancy between the apparent biologic turnover rates for endogenous gamma globulin as determined in this study, and the rates for exogenous gamma globulin as determined by others in similar patients,

spotlights a controversial aspect of all studies of this nature, namely, that the individual may metabolize exogenous foreign proteins in a different manner from endogenous native proteins.

Attempts to ascertain the mechanism producing hyperglobulinemia in multiple myeloma by studies of the die-away plots for isotopic labels have not been successful.[9, 10] An original impression of Putman[11] that the elevated globulin level might result from their accumulation because of a decreased metabolic degradation rate has not been substantiated. While the half-lives of the myeloma globulins differ, they are in the range reported for normal serum globulins.[9, 21] The turnover rates for myeloma serum proteins labeled with C^{13} glycine, N^{15} glycine, DL-glutamic acid IC^{14}, N^{15} L-aspartic acid, and I^{131} [9, 11-13, 21] are all shorter than those obtained in the present study, highlighting again the error introduced by recycling of the S^{35} label.

In the present study, evidence is presented for an accelerated synthesis rate for the abnormal myeloma serum globulins as compared to the other serum protein fractions similarly measured. Furthermore, an analysis of electrophoretic partitions of a large series of patients with multiple myeloma presenting an abnormal serum globulin demonstrates a reduction in the absolute level of all protein fractions except the alpha-1 globulins (Table II). The evidence suggests strongly that the elevated levels of abnormal serum globulins found in multiple myeloma are the result of an accelerated synthesis rate of these fractions. The depression of the synthesis rates for the other fractions can be attributed to competition by these abnormal globulins for amino acid substrates. In myeloma, then, metabolically active serum proteins are displaced by what appears to be a metabolically inactive protein. A similar conclusion has been reached by Berson and Yalow.[21]

Attention has been called to the frequency of recurrent pulmonary bacterial infections in multiple myeloma.[14, 15] These have been attributed to a decrease in the level of circulating antibodies.[14-16] Such patients retain the ability to form specific antibodies in response to antigenetic exposure but form them in inadequate amounts.[14-16] The results of the present study favor Waldenström's suggestion[17] that the pathologic globulin fractions may have a higher priority for available amino acids than normal antibodies.

Although the phrase, abnormal serum globulin, has been used throughout this paper when referring to distinctive proteins present in multiple myeloma, no evidence has been presented to indicate that these are actually abnormal proteins. The true nature of these globulins has yet to be determined.[10]

The origin and interrelationship of Bence Jones protein and the abnormal serum proteins in multiple myeloma remain unsolved. In the present study, the maximal concentration of the S^{35} label into Bence Jones protein exceeded and preceded its maximum concentration into the serum proteins. These findings are similar to those previously obtained by Putman[10] and Osserman and associates[9] and preclude the possibility that Bence Jones proteins are

derived from the serum proteins. The possibility has been suggested that Bence Jones protein is an abortive product or precursor of the serum globulin.[10] In this study, however, the formation of Bence Jones protein had no apparent effect upon the formation of serum proteins, in sharp contradistinction to the formation of abnormal serum globulins in which synthesis of other serum protein fractions was depressed. These results are in keeping with the clinical observation that the presence of Bence Jones protein is frequently associated with a normal serum protein electrophoretic pattern. This suggests that the origin of the Bence Jones protein is from a source which does not contribute to the formation of the serum proteins, i.e., that its formation and fate is independent of that of the serum proteins. While it is possible that the renal clearance of this protein is so complete that a circulating level is very low or not evident, the possibility that the kidney may be a source of Bence Jones protein should be considered.

The use of the amino acid methionine as the carrier of the S^{35} label is of special interest in the study of Bence Jones protein. A number of studies have demonstrated either absent or abnormally low methionine content in these proteins.[10] It has been suggested that Bence Jones protein is an abortive serum protein product because of a specific block in the utilization of methionine under certain conditions of accelerated protein synthesis.[18] The turnover rates for S^{35} methionine content of Bence Jones protein in this study conform well to those found for other labeled amino acids.[9, 10] There is no evidence in the one patient studied for a block in the utilization of methionine in the formation of abnormal proteins. This does not exclude the possibility that such a block may exist in other patients. Although chromatographic separation of S^{35} methionine from S^{35} cystine of Bence Jones protein was not performed, the degree of radioactivity of the protein is too great to attribute it solely to the small amount of known S^{35} cystine contaminant.

SUMMARY

The uptake and die-away plots of autosynthesized serum proteins labeled with S^{35} methionine has been presented in 3 patients with idiopathic hypogammaglobulinemia and 4 patients with multiple myeloma.

The protein defect of idiopathic hypogammaglobulinemia can be ascribed to a decreased ability to form gamma globulins. Such gamma globulin as is formed was demonstrated to have an abnormally rapid apparent turnover rate in 2 patients. Evidence was also presented demonstrating that marked discrepancies may exist in the manner in which an individual may metabolize exogenous as compared to endogenous gamma globulin.

The presence of large amounts of abnormal serum proteins in multiple myeloma could be ascribed in part to the abnormally high uptake maxima observed in these patients, which may reflect high synthesis rates and further, when compared with uptake maxima of the normal serum protein fractions in multiple myeloma, suggests serious competition by the abnormal serum protein for amino acid substrates.

In one patient, the formation of Bence Jones protein occurred independently of and had no influence upon the apparent formation of serum proteins. This suggests a separate and unrelated origin for this protein. There was no indication that a block in the metabolic pathway for the utilization of methionine was present in the synthesis and degradation of any abnormal myeloma protein.

We are grateful to Dr. S. Howard Armstrong, Jr., for his constant encouragement and many helpful suggestions for performance of this study and to Dr. Hans Popper under whose guidance the study of the serum protein electrophoretic pattern in multiple myeloma was initiated. We are also grateful to Mrs. Jane C. Arterbery for technical assistance and illustrations and to Drs. Robert Simons, Stephen Spinuzza, George G. Jackson, and Irving A. Friedman for referral of patients with idiopathic hypogammaglobulinemia.

ADDENDUM

Since submission of this manuscript, we have had the opportunity to study the apparent turnover time of exogenous pooled Red Cross gamma globulin in Patient A. R. A total of 49.5 Gm. of gamma globulin was administered intramuscularly in 9 days to this patient who weighed 65 kilograms. The serum gamma globulin level rose from 0.24 to 0.77 Gm. per 100 ml. The subsequent die-away plot, using paper electrophoretic determination of gamma globulin, showed a corrected apparent turnover time of 36 days. This should be compared to the apparent turnover time of 6 days obtained with the endogenous S^{35} label (Fig. 4). Thus, in this one individual, a marked dissimilarity has been demonstrated in the manner in which he metabolizes exogenous foreign gamma globulins as compared to his own native gamma globulins.

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THE EFFECTS OF APNEA, ENDOTRACHEAL SUCTION, AND OXYGEN INSUFFLATION, ALONE AND IN COMBINATION, UPON ARTERIAL OXYGEN SATURATION IN ANESTHETIZED PATIENTS

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IT WAS observed that prolonged, and occasionally momentary, endotracheal suction resulted in a degree of hypoxia easily detectable by inspection of the mucosae or nail beds. Since endotracheal suction as a means of maintaining a clear airway is often employed in conjunction with anesthesia, the present study was undertaken in order to determine the degree and modality of hypoxia secondary to endotracheal suction in anesthetized patients as well as to assess the feasibility of protective measures.

MATERIALS AND METHODS

Fifteen white men with no history or clinical evidence of cardiovascular or pulmonary diseases were selected for this study. They were scheduled for elective minor surgery at 9:00 A.M. Anesthesia was induced at 8:00 A.M. and all investigative procedures completed by 9:00 A.M.

After induction with intravenous Pentothal, endotracheal intubation was performed under relaxation provided by succinylcholine and the endotracheal tube was connected to a semiclosed breathing circuit. Additional amounts of Pentothal were given, as needed to maintain an adequate depth of anesthesia and to prevent bucking or coughing. Pulmonary ventilation was maintained by an Emerson ventilator cycling at +15 and −5 mm. Hg. The gas flow into the circuit was 3 liters of oxygen and 6 liters of nitrous oxide per minute in all cases.

TABLE I

A			B
A	S	O_2	NUMBER
+	0	0	13 (A)
+	+	0	12 (B)
+	0	+	11 (C)
+	+	+	12 (D)
0	0	+	48 Total
0	+	0	
0	0	0	
0	+	+	

Table I, A, shows all the possible combinations of 3 variables. A = apnea; S = suction; O_2 = oxygen insufflation. The presence of the variables is indicated by +, and absence by 0.

Table I, B, shows the type of experiments that were performed. The capital letters, in parentheses, make reference to the same letters in the text, and the numbers stand for the number of experiments in each group.

The general plan of the experiment calls for the investigation of 3 variables: apnea, suction, and oxygen insufflation. Thus a complete study should include 8 groups of experiments, each group being identified with one of the possible combinations of any 3 variables

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Received for publication Aug. 25, 1958.

as shown in Table I, A. However, one practical difficulty was encountered. In fact, while the presence of and the absence of suction or oxygen insufflation are clearly defined states, the absence of apnea may mean anything from quiet breathing to severe coughing. For this reason investigation of the 4 groups which did not include apnea has not been carried out. Fig. 1, *B* shows the 4 groups which were investigated and the number of experiments in each group.

Each experiment consisted of one minute of apnea, produced by the intravenous administration of succinylcholine. At this time the effects of the variables on arterial oxygen saturation were investigated by direct sampling and analysis. All samples were obtained at the beginning and the end of each experiment via a Cournand needle inserted in one femoral artery.

The following procedures were carried out:

A. During one minute of apnea the patient was disconnected from the ventilator and a special double-lumen catheter introduced into the endotracheal tube up to a fixed reference point. At the end of one minute the catheter was removed and artificial ventilation resumed.

B. Same as in A except that continuous suction was performed through one lumen of the catheter. Suction was measured by running it through a bellow type flowmeter and regulated to approximately 13 L. per minute.

C. Same as in A except that oxygen was insufflated at the rate of 4 L. per minute through one lumen of the catheter.

D. Same as in A except that simultaneous oxygen insufflation and suction were performed through the separate lumens of the catheter. Oxygen insufflation was limited to one-fourth to one-third of the volume of suction and never exceeded 4 L. per minute.

The number of experiments for any one patient varied from a minimum of one to a maximum of five, and the experiments were distributed among the patients in an irregular pattern. The same endotracheal tube, the same connectors, the same anesthesia machine, the same personnel, and the same double-lumen catheter were employed in all cases. The catheter was introduced for the same length in all experiments and it protruded about 2.5 cm. beyond the end of the endotracheal tube. The length of the endotracheal tube and suction catheter were such that in the average patient the tip of the catheter would lie free in the terminal portion of the trachea (Fig. 1, *A*). The ratio between the cross-section area of the double-lumen catheter and the inner bore of the endotracheal tube was 2.1 with absolute values of 33.16 and 70.84 mm.,[2] respectively. All blood gas analyses were done by the manometric technique of Sendroy[1] and Van Slyke[2, 3] as modified by Goldstein[4] and Holaday.[5] All pH determinations were done on the Model G Beckman pH meter according to the standard technique.[6]

TABLE II. ARTERIAL OXYGEN SATURATION CHANGES

VARIABLE	AVERAGE PER CENT CONTROL	AVERAGE PER CENT DROP	MEDIAN PER CENT DROP	RANGE PER CENT DROP	AVERAGE SUCTION (L./MIN.)
Apnea	98	15.0	13.5	4 to 32	
Apnea and suction	98	16.0	14.0	5 to 39	12.8
Apnea and O_2 insufflation	98	1.8	2.0	0 to 3	
Apnea, O_2 insufflation, and suction	99	5.0	3.0	0 to 19	12.3

Average per cent control is the average arterial oxygen saturation at the beginning of each individual experiment in that group. Average per cent drop, median per cent drop, and range per cent drop apply to values for each group. For further details see under Results.

RESULTS

Forty-eight experiments were carried out (Table I, B).

Group A.—One minute of apnea. There were 13 experiments in this group. Oxygen saturation drops ranged from 4 to 32 per cent, with a mean of 15 per cent and a median of 13.5 per cent (Table II).

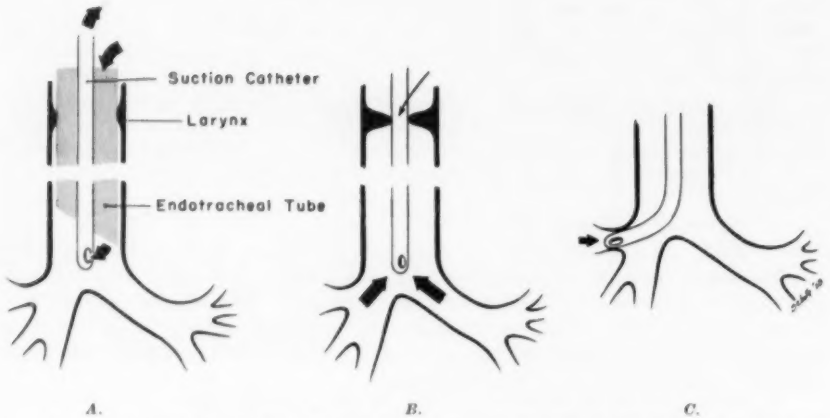

Fig. 1.—*A*, The theoretical course of events during suction of the terminal portion of the trachea through a large unobstructed endotracheal tube. *B* and *C*, Two common hazards of nasotracheal suction in clinical practice are represented diagrammatically.

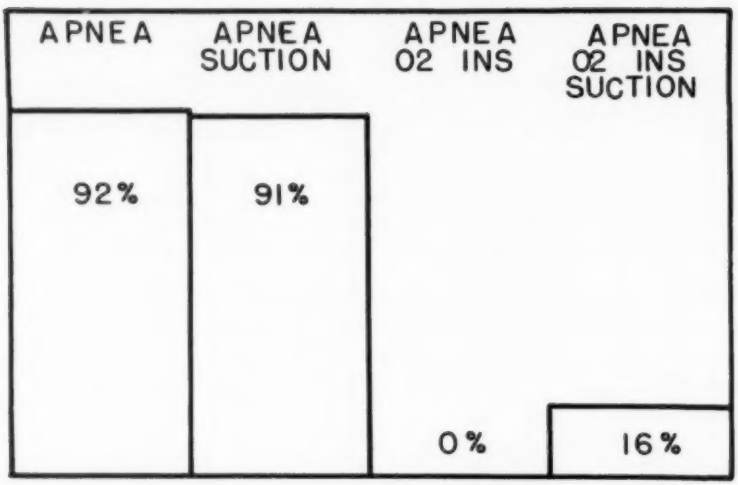

Fig. 2.—Illustrated is the incidence of critical arterial oxygen desaturation at the end of each experiment. Thus, at the end of one minute of apnea, 92 per cent of the patients were critically desaturated. At the end of one minute of apnea and endotracheal suction, 91 per cent of the patients were critically desaturated. At the end of one minute of apnea with oxygen insufflation and at the end of one minute of apnea with simultaneous endotracheal suction and oxygen insufflation 0 and 16 per cent, respectively, of the patients in each group were critically desaturated. For details on critical desaturation see text.

Group B.—One minute of apnea and suction. There were 12 experiments in this group. Oxygen saturation drops ranged from 5 to 39 per cent, with a mean of 16 per cent and a median of 14 per cent (Table II).

Group C.—One minute of apnea and oxygen insufflation. There were 12 experiments in this group. Oxygen saturation drops ranged from 0 to 3 per cent, with a mean of 1.8 per cent and a median of 2 per cent (Table II).

Group D.—One minute of apnea, suction, and oxygen insufflation. There were 11 experiments in this group. Oxygen saturation drops ranged from 0 to 19 per cent, with a mean of 5 per cent and a median of 3 per cent (Table II).

The changes observed in pCO_2 are summarized in Table III. Their importance is limited, however, and will be discussed later.

TABLE III. ARTERIAL pCO_2 CHANGES

VARIABLE	AVERAGE CONTROL (mm. Hg)	AVERAGE RISE (mm. Hg)	MEDIAN RISE (mm. Hg)	RANGE RISE (mm. Hg)
Apnea	31.8	11.0	8.7	4.2 to 34.6
Apnea and suction	34.9	13.5	12.0	6.4 to 27.9
Apnea and O_2 insufflation	35.4	14.7	15.1	7.7 to 22.9
Apnea, O_2 insufflation, and suction	31.1	12.0	12.7	4.1 to 19.0

Average control millimeters of mercury is the average arterial pCO_2 at the beginning of each experiment in that group. Average rise, median rise, and range rise apply to values for each group. See text for discussion.

DISCUSSION

Apnea of one minute's duration and apnea with endotracheal suction of the same duration appear to produce a high degree and incidence of arterial oxygen desaturation (Table II). It is interesting to observe how close the range, mean, and median values are for the two groups. Statistical comparison of the means reveals no significant difference. These findings tend to support the impression that catheter suction of the distal trachea through the usual large-bore endotracheal tube is a U-flow phenomenon interrupted only by the capture of secretions (Fig. 1, *A*).

The results observed during apnea with oxygen insufflation and apnea with simultaneous oxygen insufflation and suction are shown in Table II. The differences in average arterial oxygen desaturation and desaturation range can be demonstrated to be statistically nonsignificant. These findings offer further support to the hypothesis that in the case of endotracheal suction carried out through a large unobstructed endotracheal tube, very little air is aspirated from the lungs.

Finally, when statistical comparison is made between the groups where oxygen was insufflated and those where no insufflation occurred, a very high degree of significance is found. The idea that oxygen insufflation will maintain adequate arterial oxygen saturation in the apneic patient is not new.[7, 8] It is surprising to observe, however, how very little oxygen insufflation is needed to maintain adequate arterial saturation in the presence of simultaneous endotracheal suction.

For practical purposes it would be more interesting and convenient to know the frequency with which a given procedure is associated with a critically low arterial oxygen saturation. For the purpose of the present discussion we have accepted an arterial oxygen saturation of 93 per cent as critical. Our decision was guided by the fact that at this saturation the arterial pO_2 is 70 mm. Hg and below that the dissociation curve breaks rather sharply. If the results are tabulated following this criterion they show that endotracheal suction, as carried out in this experiment, does not significantly contribute to arterial oxygen desaturation, and that a very small amount of oxygen insufflation offers adequate protection (Fig. 2).

It is unlikely that the observed decrease in arterial oxygen saturation is secondary to diffusion anoxia.[9] Diffusion anoxia requires that the patient be sufficiently denitrogenated and that room air be inhaled to dilute the mixture present in the alveolar space. Neither factor obtained in our study. Furthermore, the reported average fall in arterial oxygen saturation at the end of one minute is 2.9 per cent[9] a figure well below ours (Table II).

The pCO_2 changes are summarized in Table III, and it can be shown that there is no statistically significant difference between any two groups. One ought to expect this to be the case since the rate of CO_2 accumulation in the blood is related, during apnea, to its rate of formation and the ability of the cells to unload it against the increasing partial pressure. The over-all rate of gain should be regulated by the respective values for output and capacity of the system, and one ought to expect it to be a logarithmic function.[10]

The clinical significance of these findings should be tempered by the recognition that apnea was a constant factor in all of these experiments, that suction was continued over a one-minute period, that the suction tip was free, and the major airways were unobstructed. Two major hazards of endotracheal suction in routine clinical practice are the possibility of wedging the tip of the catheter into one of the lesser bronchi, or clamping of the vocal cords around the suction catheter (Fig. 1, *B* and *C*). The obvious consequence of the first possibility would be failure to remove secretions at large, and possibly atelectasis of that particular portion of the lung. The second possibility presents the same hazards but on a somewhat larger scale (Fig. 1, *B*). Under these conditions the use of a double-lumen catheter for simultaneous suction and oxygen insufflation as illustrated in the present report offers some tangible and practical advantages.

SUMMARY

Under the conditions of the experiment, apnea of one minute's duration will produce severe hypoxia in all patients. The addition of endotracheal suction does not change significantly the incidence or degree of hypoxia.

Oxygen insufflation, at the rate of 4 L. per minute, will effectively prevent hypoxia both during simple apnea and during apnea with simultaneous endotracheal suction.

The clinical bearing of these findings has been discussed.

We wish to acknowledge the valuable technical assistance of Miss Margaret M. Riedy and Mr. H. E. Hodder. We wish to acknowledge also the assistance of Miss Helen Fraser in the preparation of the manuscript.

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EFFECT OF THE VALSALVA EXPERIMENT UPON ARTERIAL pCO_2, O_2 SATURATION, AND pH IN 45 PATIENTS WITH OBSTRUCTIVE PULMONARY EMPHYSEMA

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SINCE its description centuries ago by Valsalva, the Valsalva experiment[1] has been utilized sporadically by many investigators as a physiologic procedure in an attempt to evaluate the efficacy of the circulatory system in both normal and abnormal persons.[2-4] It has been used in the radiologic technique of assaying retrograde venous blood flow[5]; in the determination of venous pressure and significance of the changes in normal and abnormal persons[6]; in the determination of activity and reflex pathways of the autonomic nervous system[7-10]; and in an attempt to correlate hypoxia[11] with the syncope that sometimes develops in individuals under stress as a result of Valsalva-like maneuvers.

Our interest in the Valsalva experiment was stimulated by the incidental occurrence of syncope during performance of a vital capacity test on a patient with severe obstructive pulmonary emphysema. With the patient's cooperation, a Cournand brachial artery needle was inserted into his brachial artery and a sample of arterial blood was withdrawn as the patient attempted to perform a timed vital capacity test; each time this was done syncope developed. Subsequent analysis of the arterial blood revealed a pronounced rise in oxygen saturation, a definite decrease in pCO_2, and a concomitant rise in pH concentration. These results suggested that the syncope that frequently develops as a complication of a severe paroxysm of coughing[12, 13] or other Valsalva-like maneuver may be due to the development of acute alkalosis in addition to a decrease in cerebral blood flow and venous stasis. A study was therefore undertaken to determine the pCO_2 and O_2 saturation and the pH of arterial blood of patients with obstructive pulmonary emphysema while performing the Valsalva experiment.

This report presents the results obtained in 45 patients with mild to severe obstructive pulmonary emphysema studied in this way. Many had severe hyperinflation of the lungs with increase in total pulmonary capacity and residual air. Studies on 100 normal individuals were also done.

MATERIALS AND METHODS

A Cournand brachial artery needle was inserted into the brachial artery of one arm. Syringes that had been prepared with an internal coating of mineral oil and contained 0.75

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Received for publication May 10, 1956.
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ml. of heparin solution were used to withdraw the samples of arterial blood; following withdrawal of 20 ml. of blood (10 ml. if the Valsalva experiment was also done), a drop of mercury was drawn into the syringe for mixing purposes. The samples were then refrigerated. The O_2 and CO_2 contents were determined as soon as possible by the Van Slyke gasometric method and the pH was determined on the Beckman Model G pH meter. After a suitable rest period, with the Cournand needle in place, the first sample of arterial blood was obtained. A second sample of blood was obtained while the patient, after first breathing in the maximal amount of air, maintained a modified Valsalva experiment with oral pressure ranging between 40 and 80 mm. Hg; this second sample was obtained between the sixth and twelfth seconds of maintenance of pressure. The third sample was obtained immediately after the standard exercise test with a suitable rest period between the modified Valsalva experiment and the exercise test. All patients had the usual ventilatory tests.

TABLE I. PULMONARY FUNCTION TESTS IN NORMAL PERSONS

VALUES	MAXIMUM BREATHING CAPACITY (L./MIN.)	VITAL CAPACITY (L.)	VENTILATORY EQUIVALENT	RESIDUAL AIR (PER CENT)
Predicted Normals	100-160	3.5	2.2-2.6	20-35
(average)	114	3.4	2.4	28

TABLE II. PULMONARY FUNCTION TESTS IN GROUP II

VALUES	MAXIMUM BREATHING CAPACITY (L./MIN.)	VITAL CAPACITY (L.)	VENTILATORY EQUIVALENT	RESIDUAL AIR (PER CENT)
Predicted	100-160	3-5	2.2-3.6	20-35
Actual (average)	32	2.8	2.0	55

RESULTS

In the 45 patients with varying degrees of obstructive pulmonary emphysema, 3 types of changes in the pCO_2, O_2 saturation, and pH of the arterial blood were observed. In patients with only minimal pulmonary disease (e.g., hamartoma, isolated peripheral histoplasmosis, minimal early apical tuberculosis without evidence of obstructive pneumonitis or collapse of the involved segment or lobe) the response to the Valsalva experiment paralleled the normal. There was a rise in arterial O_2 saturation to between 96 and 100 per cent from the normal resting level of between 93 and 96 per cent, a precipitous fall in the pCO_2 to as low as 23 mm. Hg from the normal resting level of approximately 40 mm. Hg and a shift of the pH of the arterial blood to the alkaline side of normal proportionately with the fall in pCO_2, (Fig. 1). Two thirds of the 45 patients showed this normal pattern. The average values for various tests of pulmonary function are shown in Table I. The remaining third showed an abnormal pattern of one of two types. In one type there was a decrease in both arterial O_2 saturation and in pCO_2, and a rise in pH (Fig. 2). Results of analysis of pulmonary function in this group are shown in Table II. In

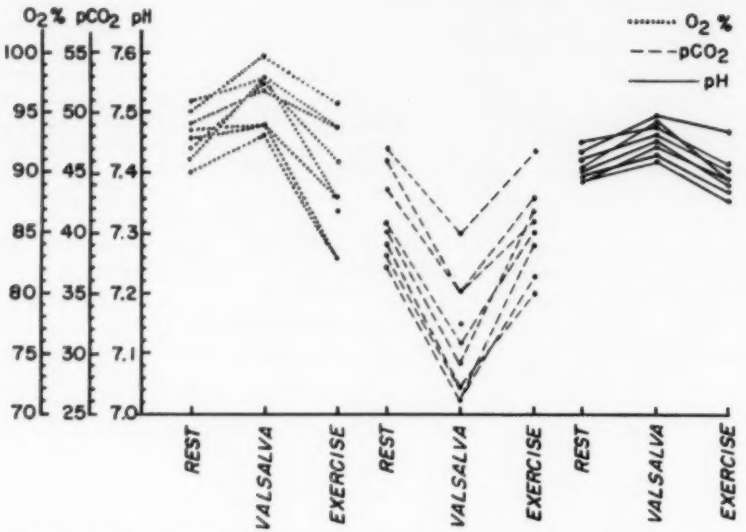

Fig. 1.—Scattergram showing the resting values and the response of pH and gases in the arterial blood during the modified Valsalva experiment and during exercise in normal persons or in those with mild to moderate obstructive pulmonary emphysema.

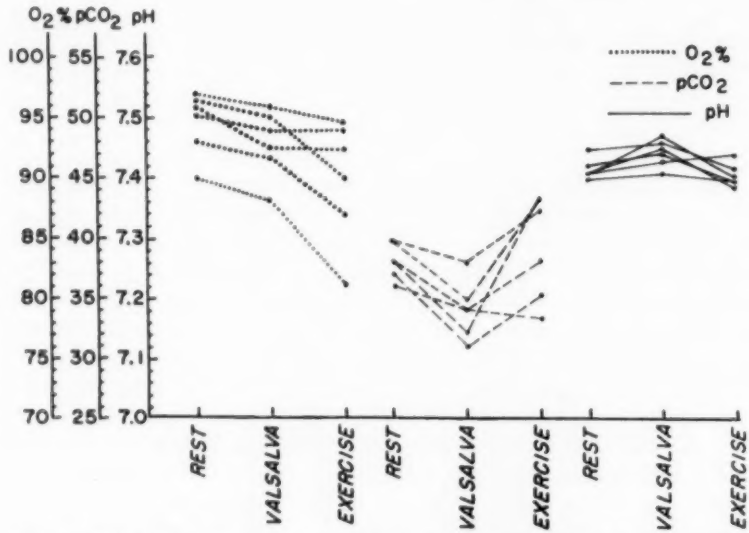

Fig. 2.—Scattergram showing the typical values and changes of the arterial blood gases and pH at rest, during the Valsalva experiment, and following exercise in patients with moderately severe obstructive pulmonary emphysema manifested by some destruction of the functional capillary pulmonary bed.

the other type there was a decrease in arterial O_2 saturation with a rise in pCO_2 and a fall in pH (Fig. 3). Table III indicates the abnormality of pulmonary function in this group.

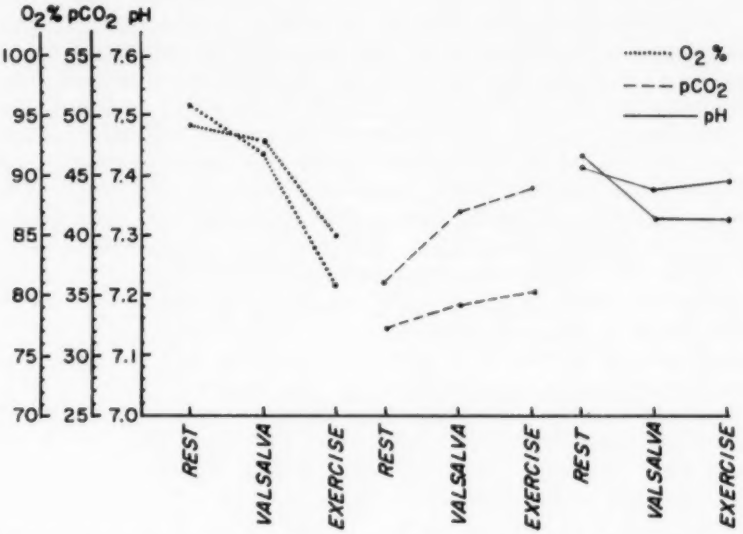

Fig. 3.—Scattergram showing the typical values and changes of the arterial blood gases and pH at rest, during the Valsalva experiment, and following exercise in patients with severe obstructive pulmonary emphysema manifested by advanced irreversible destruction of the pulmonary capillary bed.

TABLE III. PULMONARY FUNCTION TESTS IN GROUP III

VALUES	MAXIMUM BREATHING CAPACITY (L./MIN.)	VITAL CAPACITY (L.)	VENTILATORY EQUIVALENT	RESIDUAL AIR (PER CENT)
Predicted	100-160	3-5	2.2-2.6	20-35
Actual (average)	17	2.7	3.8	64

DISCUSSION

The normal response is attributed to the normal physiology of ventilation, respiration, and circulation wherein there is no disease of body tissues or other interference with the mechanics of ventilation, the diffusion aspect of the respiratory membrane, the oxygen carrying capacity of the blood, the autonomic nervous system, or the various chemoceptors and pressoceptors which are intimately associated with ventilation and respiration. In addition, normal physiology of ventilation and respiration necessitates normal function of the circulatory and cardiac apparatus.

The physiologic changes in the arterial gases during maintenance of sustained pressure in the modified Valsalva experiment with the patient in the

supine position are due to many diverse factors involving the circulatory and respiratory systems. When the normal person takes in his deepest inspiration, atmospheric air containing 20.94 volumes per cent of O_2, 0.04 volume per cent of CO_2, and 79.02 volumes per cent of nitrogen is mixed and diffused into the tracheobronchial dead space and the functioning alveolar spaces. The immediate effect is dilution of the alveolar air, which at the end of expiration contained 14.2 volumes per cent of oxygen, 5.5 volumes per cent of CO_2, and 80.3 volumes per cent of N_2.[14] This dilution increases the per cent of O_2, and decreases the per cent of CO_2 but leaves the volume per cent of N_2 fairly stable. However, within the reserve alveoli which is not normally used in quiet ventilation and normally in a state of semicollapse, but opened and distended by maximal inhalation, there is atmospheric air that is not diluted by air present after normal expiration; these alveoli, therefore, have a higher O_2 and a lower CO_2 content and tension than the alveoli used in quiet ventilation. In addition to these two factors, the maximal inspiratory effort causes increased venous return to the right side of the heart, dilatation of the right side of the heart and increase in the pulmonary capillary bed, and reflex slowing of the heart rate with consequent slowing of pulmonary blood flow. Since the alveolar oxygen tension is higher, the alveolar carbon dioxide tension lower, and the circulation through the enlarged pulmonary capillary bed is slower, there is more time for the pulmonary capillary blood to become better saturated with O_2 and for the escape of CO_2 from the blood into the alveoli.[15] Arterial blood drawn at the height of the maximal inspiratory effort showed increased O_2 saturation far above the resting level, decreased pCO_2, and shift of the pH to the alkaline side of normal.

The shift in pH to the alkaline side may be a significant factor in its effect on the increase in O_2 saturation in that alkalinity increases the affinity of hemoglobin for oxygen. If, in addition to the maximal inspiration, a modified Valsalva experiment is performed, the same mechanism first enters into play, after which the alveolar air is then placed under an oral pressure of 40 to 80 mm. Hg. This increased intrapulmonic pressure aids further in increasing arterial O_2 saturation, decreasing pCO_2, and shifting pH to the alkaline side by increasing O_2 tension when the volume of gas is compressed by contraction of the thoracic cage and elevation of the diaphragm. Venous return of greatly reduced hemoglobin from the extremities and head and neck is halted at its entrance to the thorax and abdomen[5]; this decreases the return of greatly reduced venous blood to the right side of the heart but allows visceral venous blood with less reduced hemoglobin to enter the thorax. At the same time the cardiac output is decreased and the pulmonary circulation retarded. The net result is an increased A-A gradient with diffusion of greater amounts of O_2 across the alveolocapillary membrane into the blood while the CO_2 rapidly escapes from the blood into the alveoli, owing to its greater coefficient of diffusion than that for O_2 and also to the great dilution of alveolar CO_2 by the maximal inhalation which increased the A-A gradient for CO_2. After release of the sustained pressure of the Valsalva experiment the arterial blood gases return quickly to their resting values.

It is postulated that the first type of abnormal response wherein there is a fall in both O_2 saturation and pCO_2 with a rise in pH indicates a reduction in functional pulmonary capillary bed to a degree inversely proportional to the fall in arterial pCO_2. In other words, the greater the fall in arterial pCO_2, the less destruction there is of pulmonary capillaries and therefore of pulmonary parenchyma. At this stage the aforementioned mechanisms involved in the Valsalva experiment are not able to increase the arterial O_2 saturation because of the loss of inspiratory reserve volume and reserve pulmonary capillary bed which have been destroyed by rupture and coalescence of alveoli. However, owing to the greater diffusibility of CO_2, the pCO_2 of the arterial blood decreases substantially. In addition, the presence of normal arteriovenous fistulas may allow a direct shunting of pulmonary arterial blood containing much reduced hemoglobin to the systemic circulation and this further decreases the arterial O_2 saturation of mixed arterial blood.

A fall in arterial O_2 saturation, a rise in arterial pCO_2, and a fall in pH occur as obstructive pulmonary emphysema progresses to hyperinflation of the lungs because of greater destruction of alveoli and of the pulmonary capillary bed, increased intrapleural pressure and development of pulmonary hypertension, and cor pulmonale. The mixing and diffusion of inspired atmospheric air are less effective and there is much lessened dilution of alveolar air. The Valsalva experiment with a reduced inspiratory reserve volume is less effective, owing to more numerous and larger areas of "slow space"[13] ventilation and development of bullae and blebs. The A-A gradient in "slow space" areas and large bullae favor retention of CO_2 within the pulmonary capillaries with less diffusion of O_2 into the blood from the lungs. In the more severe cases of emphysema development of an abnormal system of collateral bronchopulmonary veins[16] which anastomose with the pulmonary veins allowing backward flow of bronchial venous blood to the left side of the heart further decreases the systemic arterial O_2 saturation and increases its pCO_2 with a greater shift in pH to the acid side; greater thebesian venous drainage into the left side of the heart also contributes to hypoxia and hypercapnia.

It is believed that the modified Valsalva experiment is of prognostic value in determining the degree of pathologic changes present in the pulmonary parenchma of individuals suffering with obstructive pulmonary emphysema. These changes are irreversible if the arterial O_2 saturation fails to rise above the resting level of O_2 saturation during performance of the Valsalva experiment. On the other hand, if, with the Valsalva experiment, the arterial O_2 saturation increases above the resting level, it is believed that pulmonary disease has not reached the stage of irreversible destruction and that use of proper breathing exercises, expectorants, and bronchodilators can be expected to improve both the ventilatory and respiratory functions.

SUMMARY

Forty-five patients with varying degrees of obstructive pulmonary emphysema were studied by analysis of arterial blood gases with the patient

at rest, during a modified Valsalva experiment and following a standard exercise tolerance test. A definite relationship was noted between the values for the inspiratory reserve volume and the percentage of the predicted vital capacity and the arterial oxygen saturation and carbon dioxide tension. The Valsalva experiment can be used as a guide to the pathologic severity, reversibility, and prognosis of obstructive pulmonary emphysema.

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PULMONARY FUNCTION TESTS IN FIFTY PATIENTS WITH BRONCHIECTASIS

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ASSESSMENT of the functional capacity of the respiratory apparatus in patients with bronchiectasis should be of value in characterizing the physiologic defects resulting from bronchiectasis and determining the effectiveness of therapy in slowing or reversing these changes. Since the disease usually progresses slowly over the course of many years, any favorable effect of therapy should slow or halt this process. Evaluation of therapy by clinical criteria is difficult because it is often subjective. Obvious worsening or improvement of clinical status may take a long while and thus delay the appraisal of therapy. Objective and quantitative tests have been devised to measure various aspects of pulmonary function and are sufficiently accurate to be sensitive to changes of about 10 per cent in magnitude. It is reasonable to assume that decreases in one or more specific pulmonary functions will become evident before measurable clinical regression has occurred.

Pulmonary function tests are also important because, as early mortality in bronchiectasis is decreased by antibiotic therapy of acute infections,[24, 32] respiratory difficulty becomes a relatively more important cause of disability in patients with bronchiectasis.

As a part of a 5-year study of the natural history of bronchiectasis we have performed certain pulmonary function tests on 50 patients with this disease. Only a few studies of ventilatory function in patients with bronchiectasis have been reported and a still smaller number which include tests of distribution of gas, diffusing capacity for oxygen, and venous admixture. Furthermore, many of the patients with bronchiectasis whose pulmonary function tests have been reported in the literature have been in the younger age groups[1, 3, 4, 34, 38] whereas 24 of our patients are 50 years of age or older. We are, accordingly, reporting the results of our tests in 50 patients with bronchiectasis.

METHODS

Tests of pulmonary function were done in 32 males and 18 females, all of whom showed evidence of bronchiectasis on bronchography. Their ages ranged from 16 to 75

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This investigation was supported in part by Research Grant E-1690 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, United States Public Health Service, and by grants from the Tuberculosis Institute of Chicago and Cook County, the Charles Pfizer Company, and Lederle Laboratories Division, American Cyanamid Company.

Received for publication Aug. 29, 1958.

years with a mean age of 48 years. In 9 patients the bronchiectasis was confined to 1 lung and in 41 it was bilateral. Six patients had electrocardiographic evidence of cor pulmonale and clinical evidence of failure of the right ventricle. Two patients had hypogammaglobulinemia confirmed by repeated electrophoretic patterns obtained on paper strips.[37] One patient had situs inversus and bronchiectasis. In 20 patients emphysema was demonstrated by clinical and/or x-ray examination.

Lobectomies had been performed 3 or 4 years previously in 3 patients, in all of whom bronchiectasis had recurred. In 1 the right middle lobe had been removed; in the other 2 the left lower lobe.

No patient had had a cough for less than 2 years. Sixty per cent of the patients had had a cough for 10 or more years, 30 per cent for more than 20 years, and 14 per cent for more than 30 years.

Daily sputum volumes ranged between 0 and 500 ml. Fifty-four per cent of the patients had daily sputum volumes of 100 ml. or more, and 16 per cent had daily sputum volumes of 200 ml. or more.

The patients were classified according to the degree of dyspnea by the method of Bates and associates[5] as follows:

Class 0: Normal for man of his age.

Class 1: Capable of light work; walks one mile on the level; climbs 12 steps without undue stress.

Class 2: Capable of sedentary work; goes to and from place of work; walks up to 500 yeards on the level without stopping; can assist in household duties.

Class 3: Capable of no work; can move about the house and assist in light household duties such as laying the table.

Class 4: Dyspnea—on some days Grade 5, on others Grade 3.

Class 5: Bedridden; cannot move without dyspnea.

Thirty per cent of the patients had no complaints of shortness of breath on exertion.

None of the pulmonary function tests were performed while the patients had an acute lower respiratory tract infection. None had been confined to bed in the 2-week period preceding the day of testing.

Measurements of vital capacity and one-second vital capacity were made with a Gaensler Vitalometer (Collins).[18] The highest value of 3 trials was used. In normal individuals, the one-second vital capacity is at least 80 per cent of total vital capacity.

Maximum breathing capacity was measured by having the patient breathe as rapidly as possible into a Douglas bag, collecting the expired air, and measuring it in a Tissot spirometer. Volumes were corrected to body temperature, pressure, and saturation. Absolute values for vital capacity and maximum breathing capacity were compared to the normal values determined by Baldwin and co-workers.[38]

The air velocity index[17] was calculated by dividing the per cent of the predicted maximum breathing capacity by the per cent of the predicted vital capacity. The resulting figure was considered to be related to the velocity of expired air. Theoretically the index in normal subjects should be 1.00 ± 0.2.

The evenness of distribution of inspired gas was determined by the nitrogen washout method. Each patient breathed 100 per cent oxygen for 7 minutes. At the end of that period an alveolar sample was obtained and its nitrogen content analyzed by a Nitralyzer (Custom Engineering). In normal subjects this alveolar sample contains 2.5 per cent nitrogen[10] or less.

Measurements of venous admixture and diffusing capacity were made according to the method of Lilienthal and Riley.[21] A Riley needle was inserted into the radial artery of each subject. Blood and gas samples were collected simultaneously both while the patients breathed room air and while they breathed a mixture containing 12 to 16 per cent oxygen. Blood samples were analyzed for oxygen and carbon dioxide tension by the Riley bubble method. Partial pressure of oxygen in the expired air was measured by the

Pauling oxygen analyzer (Beckman) and by means of a Liston infrared carbon dioxide analyzer (Beckman). Each patient breathed the low oxygen mixture for at least 10 minutes until a steady state had been achieved; the second blood and gas collection was made at the end of this period. An alveolar sample obtained at the end of this period was measured for partial pressure of carbon dioxide, and this result was compared with the value obtained for partial pressure of carbon dioxide in the blood. In those cases where the values were different, the higher determination was used in subsequent calculations. The higher value of carbon dioxide tension was used because, when the distribution of blood and gas to the lungs is nonuniform, the end-tidal carbon dioxide tension may be considerably (5 to 15 mm. Hg) below the arterial carbon dioxide tension. If the alveolar carbon dioxide tension is found to be higher, the gas analyses for arterial carbon dioxide tension are assumed to be in error since it is physiologically impossible for alveolar carbon dioxide partial pressure to be greater than arterial carbon dioxide pressure except during breath holding or prolonged forced expiration. In either case, the higher carbon dioxide pressure is more likely to be the correct one.[31]

In one patient whose alveolar oxygen was already very low while he was breathing room air, a 30 per cent oxygen mixture was administered to obtain adequate alveolar and end pulmonary capillary oxygen tension necessary for this test, and the room air determinations were used for the low oxygen measurements. The difference between arterial and venous oxygen saturations was assumed to be 20 per cent for patients without cardiac disease and 40 per cent for patients with cardiac disease. The error that may be caused by these assumptions in the values obtained for diffusing capacity is small. Venous admixture and diffusing capacity were calculated graphically.[27-29] Physiologic dead space was calculated from the Bohr equation.[9] Alveolar ventilation was determined by subtracting dead-space ventilation from total ventilation.

In 3 patients the Riley needle either could not be inserted initially or became dislodged and could not be reinserted. In 12 patients duplicate analyses of oxygen and carbon dioxide failed to fall within the accepted range of ± 3 mm. Hg. These tests were discarded.[26, 33] In 7 patients alveolar-arterial oxygen tension differences were so small (below +5) as to give falsely high values for diffusing capacity. In these 7 patients, therefore, actual numerical values for diffusing capacity have not been presented.

The diffusing capacity is expressed as the number of cubic centimeters of oxygen crossing the alveolar membrane per minute per millimeter of mercury pressure gradient. In terms of body surface area the lower limit of diffusing capacity[8] is 9 ml. of oxygen per minute per mm. Hg per square meter of body surface area.

Physiologic venous admixture or shunting of blood represents that part of the blood which is not in contact with oxygenated air. The accepted upper limit of normal for venous admixture is 6 per cent.

Physiologic dead space is considered to be that portion of the expired air which does not come into contact with pulmonary blood. In normal individuals the ratio of physiologic dead space to tidal volume is 30 per cent or less. In normal individuals, arterial oxygen tension is 90 ± 5 mm. Hg or more, CO_2 tension 40 ± 2 mm. Hg,[2] and alveolar ventilation 4.2 liters per minute, uncorrected for body surface area.[9]

RESULTS

Mean values, standard deviations, and errors for all tests are given in Table I. The results of the ventilatory studies are given in Table II. Vital capacity was determined in 49 patients. In 2 patients the vital capacity was greater than 100 per cent of the expected value. In 18 patients (37 per cent) the vital capacity was 80 per cent of the expected value or greater, and in 20 patients (41 per cent) it was between 50 and 79 per cent of the expected value. In 11

of 49 patients (22 per cent) the vital capacities were between 25 and 50 per cent of the expected values. In no patient was the vital capacity less than 25 per cent of the predicted value (Fig. 1).

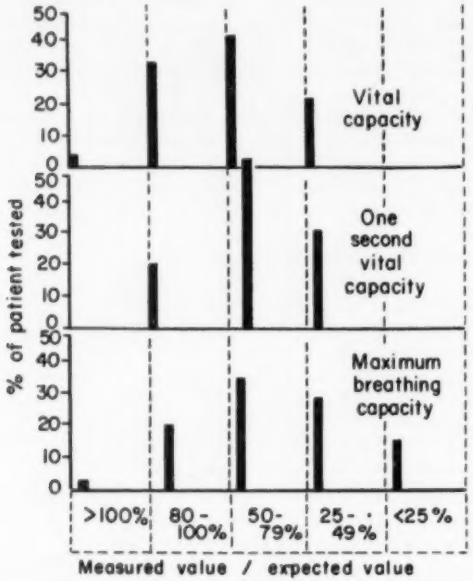

Fig. 1.—Vital capacity, one-second vital capacity, and maximum breathing capacity in 50 patients with bronchiectasis

Table I. Mean Values for Pulmonary Function Tests in 50 Patients With Bronchiectasis

TEST	MEAN ± STANDARD DEVIATION ± STANDARD ERROR
Vital capacity	69.4 % ± 19.5 ± 2.8 of expected
One-second vital capacity	63.4 % ± 18.9 ± 2.7 of total vital capacity
Maximum breathing capacity	66.6 % ± 25.6 ± 3.6 of expected
Air velocity index	0.827 ± 0.31 ± 0.04
Index of alveolar mixing	4.1 % ± 3.8 ± 0.5 alveolar nitrogen
Venous admixture	17.2 % ± 9.4 ± 1.6
Diffusing capacity	10.6 ± ml. O_2/min./mm. Hg/M^2 B.S.A. ± 4.8 ± 0.9
Arterial saturation	93.0 % ± 4.4 ± 0.7
Carbon dioxide tension	42.0 mm. Hg ± 8.5 ± 1.4
Arterial oxygen tension	74.6 mm. Hg ± 11.8 ± 2.0
Physiological dead space / Tidal volume	48.5 % ± 10.9 ± 1.8
Total ventilation	5.5 L./min. ± 1.4 ± 0.3
Alveolar ventilation	2.7 L./min. ± 0.7 ± 0.2

Timed vital capacity, expressed as the per cent of total vital capacity expired in the first second of maximal effort, was reduced in most patients. In 10 of 49 patients (20 per cent) the measured one-second vital capacity was 80 per cent or more of the total vital capacity, in 26 (53 per cent) it was between 50 and 79 per cent of the total, and in 13 (27 per cent) it was less than 50 per cent of the total. None of these patients had a one-second vital capacity less than 25 per cent of the total vital capacity.

In 17 of 50 patients (34 per cent) the maximum breathing capacity was 80 per cent of the expected value or greater. In 16 (32 per cent) the maximum breathing capacity was between 50 and 79 per cent of the expected value; in 12 (24 per cent) it was between 25 and 50 per cent of the expected value; and, in 5 (10 per cent) the maximum breathing capacity was less than 25 per cent of the expected value.

TABLE II. VENTILATION STUDIES IN 50 BRONCHIECTASIS PATIENTS

CASE NO.	SEX	AGE	B.S.A.	V.C.	% V.C.	1 SEC.	% 1 SEC.	M.B.C.	% M.B.C.	AVI	ALV. N_2
1	M	36	1.7	2.9	71	2.7	93	123	107	1.51	2.8
2	F	16	1.6	3.1	89	2.9	94	123	125	1.41	2.5
3	M	59	1.5	3.1	86	1.0	32	30	35	0.41	19.5
4	F	29	1.5	1.6	55	0.8	50	36	42	0.76	2.0
5	F	46	1.7	2.0	74	1.7	85	97	116	1.57	—
6	M	63	1.7	1.0	30	0.5	50	22	23	0.77	7.0
7	M	36	1.8	3.2	80	2.3	72	99	80	1.00	1.5
8	M	75	1.8	1.9	59	1.8	94	104	62	1.05	0.8
9	M	39	1.9	3.3	77	2.4	73	93	74	0.96	1.5
10	M	65	2.1	3.0	86	2.5	84	100	93	1.08	0.5
11	F	53	1.4	1.6	62	1.1	69	48	74	1.19	5.0
12	M	47	2.1	5.0	122	4.1	82	146	112	0.92	4.0
13	M	51	2.0	1.9	46	1.5	79	76	63	1.37	3.5
14	F	42	1.8	2.5	86	2.1	84	76	83	0.97	1.0
15	M	44	1.7	3.1	86	1.4	45	53	51	0.60	4.5
16	F	43	1.5	2.1	78	1.0	48	41	55	0.71	7.0
17	F	39	1.7	2.3	79	1.1	48	50	58	0.73	4.0
18	F	40	1.7	2.9	97	2.0	69	126	139	1.43	0.3
19	M	27	1.6	3.1	80	1.5	48	121	105	1.31	1.5
20	F	62	1.8	3.9	108	1.4	36	61	62	0.57	6.0
21	M	31	1.9	—	—	—	—	95	87	—	8.5
22	M	54	1.6	1.3	38	0.7	54	24	26	0.68	2.0
23	F	58	1.2	1.3	57	0.7	55	30	55	0.96	4.1
24	M	46	2.0	1.9	49	1.9	100	91	73	1.49	0.8
25	M	37	2.2	2.0	46	0.9	45	46	30	0.65	8.0
26	M	63	1.8	2.9	83	2.3	79	100	101	1.22	0.3
27	M	68	1.6	1.5	46	0.9	60	42	52	1.13	2.5
28	M	53	1.9	2.0	54	1.0	50	42	39	0.72	3.0
29	M	52	1.7	2.8	78	0.9	32	41	41	0.53	9.0
30	M	26	1.5	3.2	77	2.8	87	92	79	1.03	4.0
31	M	69	1.7	2.9	83	1.6	55	62	76	0.92	—
32	M	44	2.1	2.2	54	0.8	36	31	23	0.43	5.0
33	M	39	1.8	2.2	55	0.7	32	51	42	0.76	7.0
34	M	67	1.8	3.0	84	1.5	50	104	113	1.29	3.0
35	M	63	1.8	2.5	69	0.7	28	24	24	0.35	17.0
36	F	44	1.6	2.5	93	2.0	80	84	105	1.13	0.5
37	F	73	1.8	1.3	47	0.9	69	48	61	1.29	0.8
38	M	29	2.0	4.2	89	3.4	72	137	97	1.09	3.5
39	M	55	1.8	1.7	40	1.0	59	27	31	0.77	2.0
40	M	71	2.0	2.3	67	1.1	48	29	30	0.45	6.0
41	F	52	1.6	1.6	60	1.2	74	44	59	0.98	2.3
42	F	63	1.5	1.1	46	0.7	66	24	37	0.80	3.5
43	M	38	1.6	1.3	34	0.8	62	21	21	0.62	2.5
44	M	59	1.6	2.3	68	1.6	70	24	26	0.38	2.0
45	F	39	1.8	2.4	83	1.7	71	79	79	0.95	1.5
46	M	60	1.8	2.2	64	0.5	41	25	25	0.39	6.5
47	F	35	1.4	2.6	93	1.6	61	65	84	0.90	3.0
48	F	18	1.5	2.0	59	1.7	70	87	96	1.63	0.6
49	M	70	1.9	1.1	32	0.6	56	23	23	0.72	8.0
50	F	34	1.5	2.7	90	1.7	64	104	104	1.15	2.5

Key to abbreviations: B.S.A. = body surface area; V.C. = vital capacity measured, liters per minute; % V.C. = per cent of expected vital capacity; 1 Sec. = 1-second vital capacity, liters per minute; % 1 Sec. = per cent of total measured vital capacity; M.B.C. = maximum breathing capacity, liters per minute; % M.B.C. = per cent of expected maximum breathing capacity, liters per minute; AVI = air velocity index; Alv. N_2 = index of alveolar mixing, per cent nitrogen at 7 minutes.

TABLE III. ANALYSES OF BLOOD AND EXPIRED AIR FROM PATIENTS WITH BRONCHIECTASIS

CASE NO.	VE/M^2	VA/M^2	VO_2	VD/VT	ROOM AIR					12 TO 16 PER CENT OXYGEN					$\frac{\dot{Q}s}{\dot{Q}}$	PAO_2-PCO_2	DO_2/M^2
					PCO_2	PAO_2	PaO_2	A-a	SaO_2	PCO_2	PAO_2	PaO_2	A-a	SaO_2			
1	6.6	3.4	280	0.48	44	112	84	28	96	39	55	41	14	77	15	20	8
2	4.2	3.4	250	0.27	41	102	81	21	95	33	51	43	8	80	14	11	14
4	5.5	2.6	220	0.53	38	105	71	34	93	30	55	46	9	83	30	10	15
6	5.4	2.3	360	0.59	60	82	72	10	91	44	45	41	4*	73	6	*	Normal
9	6.8	3.3	330	0.49	37	112	87	25	97	37	51	36	15	70	13	18	10
10	5.5	2.7	350	0.50	40	100	87	13	96	40	49	36	13	70	8	17	10
12	4.1	2.3	340	0.43	40	98	88	10	97	38	49	51	−2*	85	6	*	Normal
13	5.9	2.6	380	0.55	51	93	73	20	92	51	62	58	4*	87	9	*	Normal
15	6.3	3.0	300	0.53	40	100	74	26	93	28	60	44	16	82	10	24	7
16	4.4	2.4	180	0.31	35	115	75	40	95	36	54	51	3*	95	25	*	Normal
17	2.0	1.6	121	0.23	30	111	66	45	94	30	59	45	14	83	35	11	7
18	6.1	3.7	250	0.39	36	113	76	37	95	31	54	45	9	82	25	13	12
20	4.2	2.2	230	0.48	42	105	75	30	94	32	62	44	18	81	21	20	6
24	5.0	1.9	310	0.63	35	82	71	11	93	36	62	46	16	82	4	23	7
25	5.5	2.2	360	0.59	54	90	57	33	86	47	68	44	24	76	32	25	7
26	4.0	2.4	270	0.40	40	100	98	2	97	43	49	42	7	73	3	11	13
27	4.6	2.0	330	0.57	44	82	60	22	87	43	36	39	−3	70	30	*	Normal
28	6.3	3.2	360	0.50	42	94	76	18	93	39	58	49	9	81	8	20	10
29	6.4	2.8	390	0.57	49	89	54	35	85	43	53	32	21	61	20	24	10
30	5.3	3.2	310	0.39	41	100	90	10	97	38	53	48	5	82	6	13	17
32	9.1	5.0	350	0.45	29	100	81	19	96	27	60	51	9	87	13	14	15
33	7.0	4.0	310	0.43	28	115	84	31	98	29	60	35	25	72	14	28	7
34	7.5	3.4	480	0.54	35	97	85	12	97	36	57	47	10	83	32	15	18
35	7.9	3.8	400	0.51	37	104	68	36	93	35	72	50	22	84	15	25	9
36	3.6	2.7	217	0.26	31	103	86	17	97	40	45	41	4*	75	9	*	Normal
37	5.8	2.6	270	0.55	44	100	99	9	97	34	55	35	22	70	2	25	11
39	5.5	1.9	250	0.65	46	93	62	31	91	43	65	45	20	80	30	23	6
40	5.1	2.6	380	0.52	54	100	77	23	93	46	66	57	9	87	18	12	16
42	4.1	2.0	240	0.50	50	88	66	22	90	46	52	32	20	79	21	20	8
43	6.4	2.5	210	0.61	53	100	65	35	91	50	56	30	26	75	30	25	5
45	4.6	2.0	300	0.57	44	93	72	21	93	42	62	54	8	85	19	13	13
46	5.0	2.0	320	0.59	56	101	65	36	88	56	101	65	36	88	31	10	18
47	5.1	2.7	310	0.46	41	100	67	33	91	44	40	36	4	65	29	*	Normal
49	5.6	4.3	390	0.60	60	141	82	59	94	60	91	47	44	76	24	50	4
51	4.4	3.0	200	0.29	31	112	80	32	96	32	57	42	15	78	19	16	8

Key to abbreviations: VE/M^2 = total ventilation per square meter of body surface area; VA/M^2 = alveolar ventilation per square meter of body surface area; VO_2 = volume oxygen consumed, room air; VD/VT = physiologic dead space/tidal volume ratio; PCO_2 = partial pressure CO_2; PAO_2 = partial pressure alveolar O_2; PaO_2 = partial pressure arterial O_2; A-a = alveolar-arterial PO_2 difference; SaO_2 = saturation; $\frac{\dot{Q}s}{\dot{Q}}$ = venous admixture; PAO_2-PCO_2 = alveolar-mean capillary oxygen tension; DO_2/M^2 = diffusing capacity per square meter of body surface area.

*A–a too small for accurate calculations.

In 20 of 49 patients (41 per cent) the air velocity index was greater than 1.0, while in 23 of 49 (47 per cent) it was between 0.5 and 1.0. In an additional 6 patients (12 per cent) the air velocity index was less than 0.5.

Reduction of maximum breathing capacity was associated with reductions in vital capacity, timed vital capacity, and air velocity index. The correlations between maximum breathing capacity and these other 3 factors were high, with a p value of 0.01 or less calculated by the chi-square method.

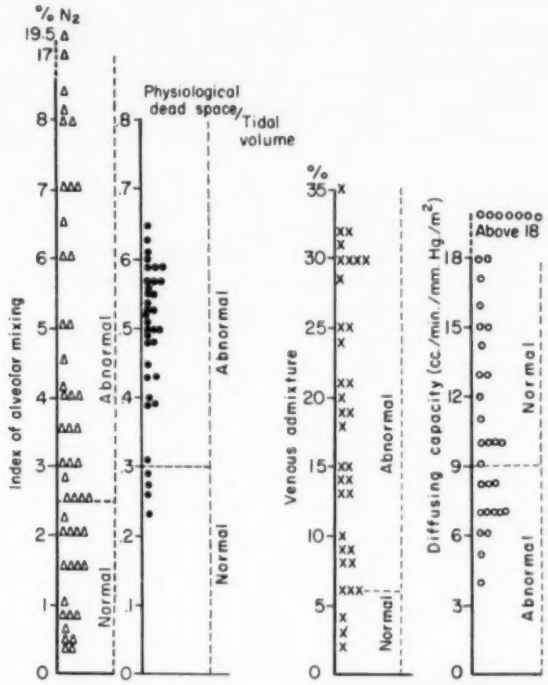

Fig. 2.—Distribution of values attained for index of alveolar mixing, physiologic dead space–tidal volume ratio, venous admixtures, and diffusing capacity in patients with bronchiectasis.

The index of alveolar mixing was determined in 48 patients. Fig. 2 shows the distribution of results in this test. The mean value of the entire group was well above normal limits. In 26 patients (53 per cent) the index was over 2.5 per cent and in 16 (33 per cent) it was over 4 per cent. One patient had 19.5 per cent nitrogen and another had 17 per cent nitrogen in his alveolar sample after 7 minutes.

The results obtained in blood and gas studies for each patient are given in Table III. In 31 of 35 patients (89 per cent) the total dead space to tidal volume ratio was greater than 30 per cent (Fig. 2); in 17 (49 per cent) it was greater than 50 per cent, and in 3 (9 per cent) this ratio was greater than 60

per cent. Increased dead space correlated well with decreased vital capacity ($p = 0.01$). Despite the increased dead space, alveolar ventilation in these patients was normal, total ventilation being somewhat increased.

In 4 of 35 patients the arterial saturation was below 90 per cent; in 2 it was below 87 per cent. This is much below the lower limit of normal of 92 to 93 per cent. The mean arterial oxygen tension for the group was also reduced. In 2 of 35 patients the arterial tension was below 60 mm. Hg while the patient was breathing room air.

The mean arterial carbon dioxide tension was within the upper limits of normal. However, in 7 patients (20 per cent) the arterial CO_2 tension was above 50, indicating significant ventilatory insufficiency.

In only 6 of 35 patients (18 per cent) was the venous admixture, as calculated, within normal limits. In 24 patients (68 per cent) it was greater than 20 per cent.

While increased venous admixture was a common abnormality, decreased diffusing capacity was not. In 23 of 35 patients (66 per cent) the diffusing capacity was within normal limits. No patient had a diffusing capacity less than 4.0. Of the 12 patients with abnormally low diffusing capacity, 10 also had an abnormally high degree of venous admixture.

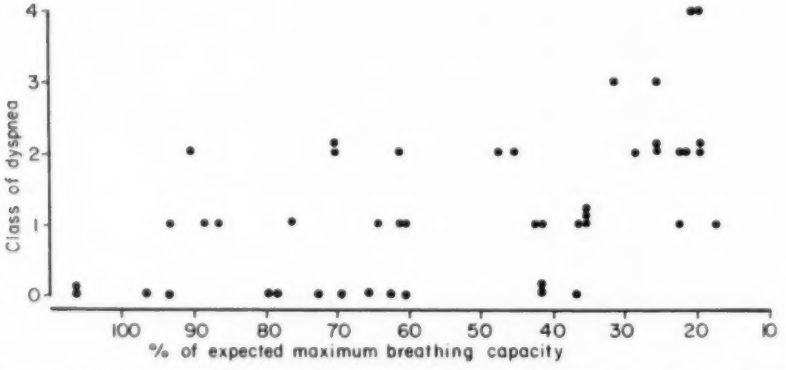

Fig. 3.—Per cent of predicted maximum breathing capacity compared to class of dyspnea for patients.

In a group of patients with known severe emphysema, Donald[11] plotted effective alveolar-mean pulmonary capillary pressure against venous admixture and dead space. He found that increased venous admixture and dead space occurred in those patients with the greater alveolar capillary gradients. Thus, he concluded that the diffusion defect that had been recorded in these patients was only apparent and was caused by a decrease in the total area of pulmonary membrane available for diffusion. However, in the present study little correlation was demonstrated between venous admixture, dead space, and increased alveolar-mean capillary gradients.

Tests of correlation of each parameter of pulmonary function tested in the group were performed against grade of dyspnea, duration of illness, age, and sputum volume. No significant correlations were found except between maximum breathing capacity and dyspnea (Fig. 3). In this case the p value was less than 0.01 by the chi-square method.

DISCUSSION

Studies of pulmonary function in patients with bronchiectasis have been performed by Smith and associates,[34] by Anderson and associates,[1] and by Williams.[38] Their results are compared with the results obtained in this study in Table IV. In addition, Baldwin and associates[3, 4] examined 3 patients with bronchiectasis and found ventilatory defects in all 3, and decreased arterial saturation in one. Their studies are not included in the table owing to insufficient details in their report.

In all but 2 of our patients some defect of pulmonary function was uncovered. A high percentage of our patients had ventilatory defects and increased physiologic dead space to tidal volume ratios.

Smith demonstrated that the ventilatory deficiency in bronchiectasis is proportional to the extent of the disease as shown by the decreased measured vital capacities and maximum breathing capacities found in these patients. Resection was shown by him to produce a further ventilatory loss proportional to the volume of the lung resected.

In comparing our results with those of others it should be emphasized that almost all of our patients had extensive bilateral bronchiectasis and had experienced some degree of dyspnea on moderate exertion for many years. If Smith's conclusion is correct, one would expect to find marked impairment in pulmonary function in many of our patients and this was indeed the case. Although our group was not typical and was considerably weighted by having so few patients with early bronchiectasis, it is precisely in these more severely affected patients that surgical resection is most difficult and least satisfactory, and it is they who must rely on medical management.

Many factors besides disease can cause apparent defects in pulmonary function as measured by currently used tests. For instance, it is thought that after prolonged bed rest small areas of atelectasis may occur and pulmonary function may be decreased. Although half of our patients were unable to support themselves entirely, none was confined to bed and all engaged in at least a moderate amount of activity.

Excessive smoking, liver disease, and other chronic diseases may alter pulmonary function.[7, 16] However, there is no evidence that these occurred more frequently in our patients than in a group of subjects without bronchiectasis in a similar age range.

Half of our patients were over 50 years of age and it is known that aging in itself can depress pulmonary function. It is likely that aging tended to increase the abnormalities measured in these patients. Tenney[36] in a study of

TABLE IV. REPORTS OF ABNORMAL PULMONARY FUNCTION IN PATIENTS WITH BRONCHIECTASIS

REFERENCE	V.C.*	PER CENT ABNORMAL†	M.B.C.*	PER CENT ABNORMAL†	VENOUS ADMIXTURE*	PER CENT ABNORMAL‡	DO_2*	PER CENT ABNORMAL‡	DEAD SPACE* / TIDAL VOLUME	PER CENT ABNORMAL‡	NO. OVER FIFTY
Smith et al.[34]	64	9	64	9	10	60	0	—	10	30	2
Anderson et al.[1]	5	—	5	60	5	60	5	60	0	—	2
Williams[38]	6	50	6	67	6	100	6	33	6	33	0
Present study	49	63	50	78	35	82	35	34	35	89	24

Key to abbreviations: V.C. = vital capacity; M.B.C. = maximum breathing capacity; DO_2 = diffusing capacity.
*Number of patients tested.
†Per cent with actual value less than 80 per cent of predicted values.
‡Per cent abnormal according to the criteria of each author.

18 normal men over 68 showed that mean anatomic dead space was increased but that the range of variation was great. Greifenstein[19] studied lung function in 11 men and 10 women over 50 years of age with no apparent pulmonary disease. The mean age of his group was 61.5 years. Although he found abnormalities of arterial saturation, gas distribution, and ventilatory function in some, the abnormalities he discovered were not so great nor did they occur in so high a percentage of his total group as did the abnormalities demonstrated in our group of patients with bronchiectasis. The mean arterial saturation for Greifenstein's group of 21 patients over the age of 50 was 96.5 per cent compared to 93.0 per cent for our patients. In only one of his patients was the arterial saturation below 93. Mean values for the washout of nitrogen were 1.57 per cent after 7 minutes for men and 1.1 per cent for women. Only two of his patients showed an abnormal value for this test. It is difficult to compare Greifenstein's results for vital capacity and maximum breathing capacity with our own, since values for individual patients are not given and his results are expressed in terms of absolute values only.

Moreover, there was no correlation between age and degree of impairment of any of the pulmonary function tests in our patients; this apparently indicated that the extent of disease process rather than aging was the more important factor.

Baldwin has defined two kinds of pulmonary decompensation: (1) ventilatory insufficiency, and (2) alveolar-respiratory insufficiency. In ventilatory insufficiency the defect is primarily in the bellows action of the lungs and is reflected in abnormalities in the vital capacity, the one-second vital capacity, and the maximum breathing capacity. In alveolar-respiratory insufficiency there are defects in diffusion and distribution of blood and gas. Defects in the latter group are reflected as physiologic abnormalities in intrapulmonary mixing, diffusion, dead space, and venous admixture.

In our series most patients had abnormalities in both these groups of tests and the vast majority had multiple abnormal tests.

The relative importance of the different mechanisms by which such marked alterations in pulmonary physiology are produced in bronchiectasis cannot be clearly delineated. Excessive secretions and bronchospasm undoubtedly hinder the movement of air into and out of the lungs, thereby decreasing measured values of timed vital capacity and maximum breathing capacity. Pulmonary fibrosis and pleural adhesions following repeated attacks of bronchopneumonia and pleural effusion can seriously limit vital capacity and decrease diffusion from alveoli to capillaries. Similarly, small areas of atelectasis and emphysema can cause maldistribution of gas to alveoli. Communications between bronchial and pulmonary arteries can increase dead space and decrease diffusing capacity. Dilatation of the bronchi can also increase dead space and pulmonary fibrosis may decrease diffusing capacity.

Obstruction to air flow and maldistribution of gas and blood, as evidenced by increased dead space and venous admixture, were particularly striking in

our patients. Maximum breathing capacity was reduced more than vital capacity in some patients. Therefore, the air velocity index was also reduced. Gaensler[17] has shown that the index is more than 1 in patients with diseases which restrict the movement of the lungs, as in pulmonary fibrosis. When the index is less than 1, the disease is primarily obstructive as in asthma or emphysema. In our patients, the mean was 0.83 and in 12 per cent of the cases the index was less than 0.5.

Venous admixture can be divided into that part caused by true or anatomic communication, as may occur between the bronchial and pulmonary veins, and virtual or physiologic venous admixture which occurs when blood perfuses poorly ventilated alveoli. True anatomic shunting between bronchial and pulmonary veins occurs particularly in localized areas of emphysema and this may account for part of the increased shunting that was measured in these patients. Virtual shunting probably played an even greater role. When secretions prevented air from entering certain alveoli, pulmonary capillary blood perfusing these alveoli could not be oxygenated and would contain only venous concentrations of oxygen.

Venous admixture, although already greatly increased in these patients, might have been even greater if it were not for communications that are said to exist between bronchial and pulmonary arteries in bronchiectasis. Liebow[6, 20] has demonstrated these communications anatomically in patients with this disease. Since the blood in the bronchial arteries is at systemic pressure, some of it flows in these communications from bronchial to pulmonary artery. This flow probably serves to divert pulmonary blood from flowing through diseased areas and thereby tends to decrease the amount of physiologic venous admixture. Roosenburg and Deenstra[30] by catheterization studies demonstrated increased oxygen tension in blood from the pulmonary arteries of patients with bronchiectasis. Fishman[13] found pulmonary collateral blood flows of 1 liter per minute in subjects with bronchiectasis.

Dead space is considered to be that portion of the inspired air which does not come into contact with flowing blood. Like venous admixture it is divided into physiologic and anatomic types. Anatomic dead space consists of the volume of inspired gas which cannot, for anatomic reasons, contact pulmonary capillary blood. It consists for the most part of the gas in the trachea and bronchial tree. Physiologic dead space, on the other hand, is caused by an abnormal decrease in the perfusion of pulmonary capillaries or an abnormal increase of ventilation in a normally perfused area.

The increased dead space found in 89 per cent of the patients studied could be caused by increased volume of the tracheobronchial tree or the ventilation of poorly perfused alveoli. Considering the dilatations of the bronchi which are found in this disease the former explanation seems far more likely. Emphysematous changes in the lungs probably contributed to the increased dead space in some patients, but in the majority the dead space was increased without evidence of emphysema.

Another factor that may contribute to increased effective dead space in these patients is the arterial blood flowing from the bronchial arteries through the pulmonary capillaries. This left-to-right shunt of blood which has already passed through pulmonary capillaries reduces the gradients of gas tension across the alveolar-capillary membrane. The result is decreased gas exchange in these alveoli and a small contribution to physiologic dead space.

For the same reason arterioarterial shunting would decrease diffusing capacity. Since little or no diffusion could take place when oxygen tension in the alveoli was at the same tension as bronchial arterial blood the total effective diffusion would be decreased and diffusing capacity would be decreased when it is measured for the total lung as in the Riley method. Numerous bronchospirometric studies in patients with bronchiectasis have shown little or no oxygen uptake from diseased areas. Roosenburg[30] believes that in extreme cases oxygen may even diffuse into the alveoli from the blood.

Studies by Marks,[22] Austrian,[2] and Donald[11] have shown that increased venous admixture is often associated with decreased diffusing capacity. Donald and associates[11] have stated that in patients with severe disease of the lung it is difficult to distinguish venous admixture from defects of diffusion.

Decreased diffusing capacity occurs in two situations: (1) When the total effective area available for diffusion is decreased, and (2) where the alveolar-capillary membrane is so altered that diffusion through it is impeded.

In chronic disease states of the lung where some alveoli are ventilated and not perfused and some perfused and not ventilated, the total effective area for diffusion will be decreased without any real change occurring in the alveolar-capillary membrane. The fact that we find no relationship between venous admixture, dead space, and effective alveolar-mean capillary oxygen gradient indicates that perhaps in some of our cases of bronchiectasis enough changes had occurred in the alveolar-capillary membrane to cause a primary defect in diffusion.

SUMMARY

Vital capacity, one-second vital capacity, maximum breathing capacity, air velocity index, index of alveolar mixing, physiologic dead space, venous admixture, and diffusing capacity were determined in 50 patients with bronchiectasis confirmed by bronchography.

At least one of these tests was abnormal in 48 patients and more than one test was abnormal in nearly every patient.

In 63 per cent of the patients tested the vital capacity was less than 80 per cent of the expected value; in 66 per cent the maximum breathing capacity was less than 80 per cent of the expected value; and in 80 per cent the one-second vital capacity was less than 80 per cent of the total vital capacity.

The index of alveolar mixing was greater than 2.5 per cent in 53 per cent of the patients in this series, and the air velocity index was less than 1 in 59 per cent.

The physiologic dead space was greater than 30 per cent of the tidal volume in 89 per cent; venous admixture was greater than 6 per cent in 82 per cent; and diffusing capacity was less than 9 ml. of O_2 per minute per millimeter of mercury per square meter in 34 per cent of the patients tested.

Maximum breathing capacity correlated well with vital capacity, one-second vital capacity, and air velocity index. Maximum breathing capacity also correlated well with degree of dyspnea as judged by the patients' histories. Vital capacity and physiologic dead space were also closely correlated with tidal volume.

The relationship of these physiologic abnormalities to the disease, bronchiectasis, has been discussed.

We wish to thank Drs. Joyce Z. Pearson and Ronald E. Fox for clinical assistance and Miss Margaret Mellody, Miss Mae Hasegawa, Miss Jean Jarnez and Mr. Carell Hutchinson for technical assistance.

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HUMAN THROMBOCYTES AND LEUKOCYTES AS A SOURCE OF SOME OF THE ACID MUCOPOLYSACCHARIDES OF SERUM

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IN THE course of recent studies of acid mucopolysaccharide levels in human plasma[1] it was observed that levels of hexuronate-containing polysaccharides in human plasma were considerably lower than those reported in human serum by Bollet and associates.[2] This discrepancy had been noted previously by Bollet,[3] and the higher levels in human serum were confirmed subsequently in our laboratories. The present study was initiated to compare levels in simultaneously drawn plasma and serum samples, using the method of Bollet and co-workers for recovery of the acid mucopolysaccharides.[2] If the discrepancy in levels was again observed, a further objective was to determine its source.

Serum levels consistently exceeded plasma levels provided that the clotting process proceeded unhampered and provided also that the usual cellular components were present in the whole blood. Acid mucopolysaccharides in human leukocytes have been reported previously.[4, 5] The finding of acid mucopolysaccharides in thrombocytes has been reported in rats.[6] The present study demonstrated the occurrence of acid mucopolysaccharides in human thrombocytes also. The over-all findings suggested that leukocytes and perhaps thrombocytes were at least one source for the higher level of acid mucopolysaccharides in human serum as compared to plasma.

METHOD

Blood was obtained by venipuncture from hospitalized patients, using siliconized syringes and a rapid withdrawal technique. The blood was dispensed promptly in approximately 5 ml. amounts to a series of nonsiliconized 13 by 100 mm. glass tubes, either empty or containing 5 mg. dry potassium oxalate and 7.5 mg. dry ammonium oxalate. This constituted the usual and standard method for obtaining plasma for the studies reported here (including Table I and Table II, A and C). For particular studies (Table II, B), blood was permitted to stand briefly before being oxalated so that, after tubing, spontaneous sedimentation still occurred but without evidence of surface buffy coat when centrifuged, indicating that early and incomplete clotting had trapped these elements in the erythrocyte column. The purpose of this was to see if the early stages of clotting produced the same elevation of acid mucopolysaccharide level as did complete clotting. In other studies (Table II, D), siliconized tubes were used for collection of blood both for serum and for oxalated plasma. The blood for serum was then permitted to stand at room temperature as long as was necessary for clotting and retraction to occur,

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This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service, and the American Heart Association.
Received for publication Sept. 9, 1958.

before removal of serum. In other studies not tabulated (see Results), blood was obtained simultaneously for multiple lots of serum and was permitted to stand at room temperature for varying periods of time before removal of serum from the clot.

The blood was obtained variously from patients with no demonstrable inflammatory diseases, with inflammatory diseases unrelated to connective tissue disease, with connective tissue disease, and with leukopenia (with or without accompanying connective tissue disease).

Acid mucopolysaccharides were recovered from plasma or serum by the method of Bollet and co-workers[2] and were measured by the carbazole method of Dische.[7]

Thrombocytes were separated from blood by the method of Dillard and associates.[8] Acid mucopolysaccharides were recovered by application of the method of Bollet and co-workers.[2] Paper chromatographic studies of the hexuronate-containing polysaccharides recovered from thrombocytes employed methods previously reported.[5] Quantitative and qualitative studies of the hexuronate-containing polysaccharides* in thrombocytes are in progress and will be reported later.

TABLE I. RATIO* OF ACID MUCOPOLYSACCHARIDES OF SERUM TO THOSE OF SIMULTANEOUSLY OBTAINED PLASMA

PATIENT (SEX, AGE, AND DIAGNOSIS)	C-REACTIVE PROTEIN	RATIO
F. 24; headaches	0	107
F. 32; headaches	0	116
M. 19; subacute bacterial endocarditis, treated	0	121
M. 54; peptic ulcer	0	123
M. 22; endophthalmitis, treated	0	160
M. 43; ruptured nucleus pulposus	0	163
M. 66; retinal detachment	0	166
M. 48; epilepsy	0	194
M. 54; rheumatoid arthritis	3+	132
F. 50; pneumonitis	6+	135
M. 54; tetanus; urinary tract infection	4+	150
F. 39; acute rheumatic fever	8+	151
F. 24; bronchiectasis	4+	171
F. 67; degenerative and rheumatoid arthritis	4+	197
M. 59; chronic lung disease	4+	199

* $\left(\frac{\mu g/100 \text{ ml. serum}}{\mu g/100 \text{ ml. plasma}}\right) \times 100.$

RESULTS

The data are reported as the ratio of serum acid mucopolysaccharides to plasma acid mucopolysaccharides times 100.† Table I summarizes the data obtained by the standard method first outlined, using patients with no hematologic abnormalities. It will be noted that in 13 of the 15 patients studied, the serum level exceeded the plasma level by 20 per cent or more.‡

With one exception, a small group of patients with leukopenia showed little difference between plasma and serum levels (Table II, A). This was noted also when leukocytes were removed from plasma by allowing blood to

*Preliminary studies indicate a general order of magnitude in normal individuals of 40 to 50 μg per total thrombocytes in 100 ml. blood as compared to a general order of magnitude of about 15 to 20 μg per total leukocytes in 100 ml. blood,[2] all measured as hexuronic acid. It would appear, however, that this is subject to variation in disease states, and further comment must await extension and report of current investigations.

†Absolute levels of plasma acid mucopolysaccharides determined in this laboratory, using identical methods and comparable groups of patients, have been reported in detail previously.[1]

‡The maximal cumulative error accepted in the methods used was 10 per cent. All determinations were run in duplicate as reported previously,[1] and results were discarded if the duplicates failed to check within 10 per cent.

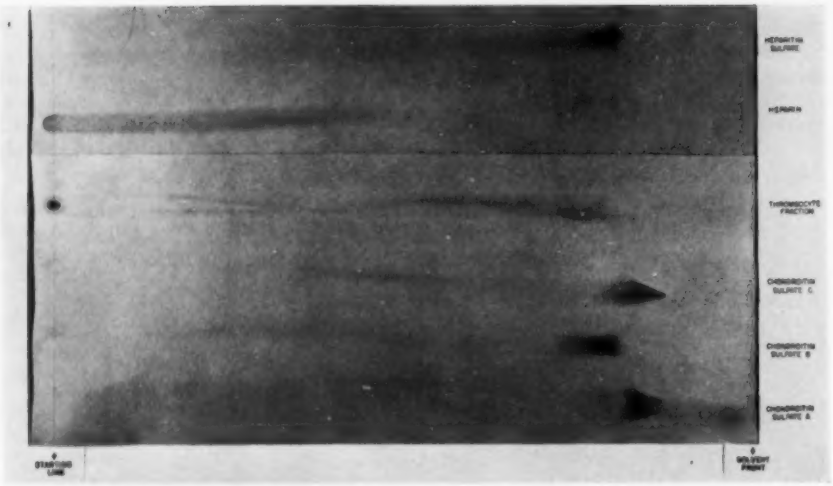

Fig. 1.—Chromatographic comparison of human thrombocyte fraction with chondroitin sulfate A, B, and C, heparin, and heparitin sulfate. Solvent 50 per cent ethanol in pH 6.8 M/15 phosphate buffer.

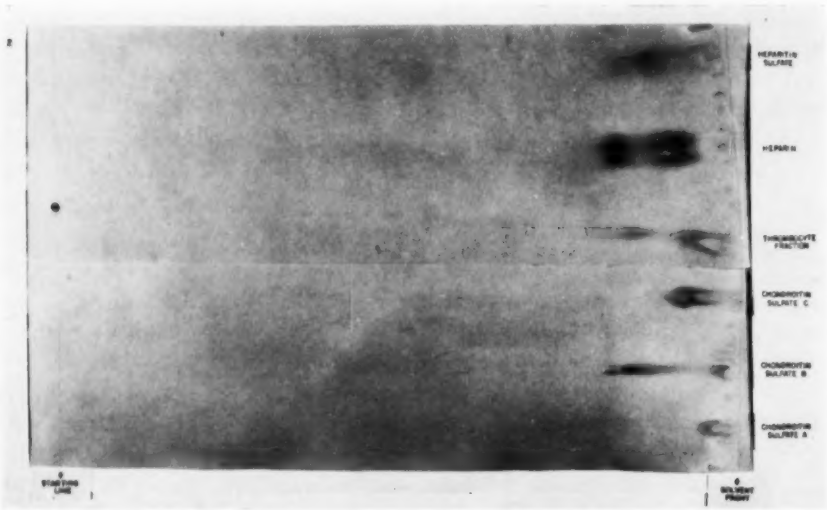

Fig. 2.—Chromatographic comparison of human thrombocyte fraction with chondroitin sulfate A, B C, heparin, and heparitin sulfate. Solvent 38 per cent n-propanol in pH 6.8 M/15 phosphate buffer.

stand very briefly before adding oxalate; although no grass clotting occurred, the leukocytes were trapped in the erythrocyte column; the acid mucopolysaccharide content of the supernatant plasma then equaled that of the corresponding serum taken off grossly clotted blood (151 and 139 μg per 100 ml. serum, respectively; Table II, B), incomplete and early clotting thus having appeared to produce the same elevation of acid mucopolysaccharide level as did complete clotting. Defibrinated plasma did not vary from plasma in acid mucopolysaccharide content (Table II, C). Serum from blood permitted to clot in siliconized glass tubes was variable in content as compared to serum collected in uncoated glass tubes, the latter twice equaling and twice considerably exceeding the former (Table II, D).

TABLE II. RATIO* OF ACID MUCOPOLYSACCHARIDES OF SERUM TO THOSE OF PLASMA IN VARIOUS SITUATIONS AFFECTING BLOOD COAGULATION OR LEUKOCYTE CONTENT

	PATIENT (SEX, AGE, AND DIAGNOSIS)	VARIANT SITUATION†	C-REACTIVE PROTEIN	RATIO
A.	M. 35; drug reaction suspected	W.B.C. 3,850 with 44 per cent P.M.N.‡	0	106
	M. 48; Felty's syndrome	W.B.C. 1,350 with 16 per cent P.M.N. On prednisone	5+	104
		W.B.C. 1,850	7+	179
	M. 29; Hodgkin's disease	W.B.C. 600 with 70 per cent P.M.N.; thrombocytes 7,000. On nitrogen mustard	6+	89
	M. 46; leukemia, probably myelocytic	W.B.C. 1,400 with 45 per cent P.M.N. On 6-mercaptopurine	4+	88
B.	M. 62; folliculitis	Delay before oxalating blood§ for plasma	0	92
C.	M. 43; ruptured nucleus pulposus	Standard plasma method§	0	163
		Plasma defibrinated with thrombin		150
D.	M. 59; chronic lung disease	Ratio is that of serum from glass tubes to serum from siliconized glass tubes	4+	102
	M. 40; rheumatoid arthritis	Ratio is that of serum from glass tubes to serum from siliconized glass tubes	6+	99
	M. 57; scleroderma	Ratio is that of serum from glass tubes to serum from siliconized glass tubes	3+	138
	M. 54; rheumatoid arthritis	Ratio is that of serum from glass tubes to serum from siliconized glass tubes	3+	135

*Ratio: $\frac{\mu g/100 \text{ ml. serum}}{\mu g/100 \text{ ml. plasma}} \times 100$, except as otherwise stated.
†Thrombocytes normally present except as noted.
‡P.M.N. = polymorphonuclear neutrophil leukocytes.
§See Methods.

Serum separated from the clot after 1.5, 4.25, and 8.25 hours of standing at room temperature showed no significant differences in content of acid mucopolysaccharides.

Hexuronate-containing polysaccharides were recovered from human thrombocytes. Chromatographically on paper the major metachromatic* component showed a mobility similar to that of either chondroitin sulfate B or heparitin sulfate (Figs. 1 and 2).† The chromatographic techniques used did

*Toluidine blue stain. The dark spot at the origin of the thrombocyte fraction (Figs. 1 and 2) was not metachromatic. All other material visible on photographs was metachromatic.

†The author is indebted to Dr. Karl Meyer for his kindness in furnishing samples of the purified acid mucopolysaccharides used as standards in the chromatographic studies.

not differentiate sufficiently between these two substances to permit closer definition, nor did they establish identity of the thrombocyte fraction with either chondroitin sulfate B or heparitin sulfate. Other techniques are in progress and will be reported on later.

Plasma remaining after removal of thrombocytes could be compared only with plasma from blood similarly anticoagulated with disodium ethylenediaminetetraacetate. In one patient comparison of levels of hexuronate-containing polysaccharides in plasma obtained by centrifuging whole blood with levels in the same plasma from which intact thrombocytes were first removed by the method of Dillard and associates[8] yielded a ratio of 156 (223 and 135 μg per 100 ml. plasma, respectively). Comparison of levels in plasma obtained by centrifuging whole blood with levels in the same plasma to which additional autologous platelets were added after centrifuging showed essentially equal levels (223 and 209 μg per 100 ml. plasma, respectively).

DISCUSSION

The demonstration of acid mucopolysaccharides in human thrombocytes was an expected event in view of the prior demonstration of such substances in the thrombocytes of rats.[6] Coupled with the prior demonstration of acid mucopolysaccharides in human leukocytes,[4, 5] this suggested two possible sources for the increased amounts of acid mucopolysaccharides found by the method of recovery of Bollet and co-workers[2] in human serum as compared to plasma. The studies reported here demonstrated that the higher level in serum was dependent on the normal occurrence of at least the early phase of the blood-clotting process and that the level was not increased further by allowing the blood to stand once clot retraction had commenced. The higher level was not dependent on the formation of a fibrin clot per se, since production of a fibrin clot by addition of thrombin to plasma did not increase the yield of acid mucopolysaccharides. The studies suggested that trapping of leukocytes and perhaps thrombocytes in the clot was necessary for production of a serum level of acid mucopolysaccharides in excess of the corresponding oxalated plasma level.

Since it has been shown[1] that the elevation of acid mucopolysaccharides in human plasma in various inflammatory states is an acute phase response, the possibility of a relationship between blood clotting and the production of acute phase reactants was attractive. A preliminary survey of the level of certain other acute phase reactants (C-reactive protein and sialic acid) has thus far failed to support such a hypothesis. The possibility is still receiving consideration, however, and is under investigation. Further studies both qualitative and quantitative of the hexuronate-containing polysaccharides of human thrombocytes are also in progress.

SUMMARY

1. The level of acid mucopolysaccharides recovered from human serum, using the method of recovery of Bollet and co-workers,[2] usually considerably exceeded that of the corresponding oxalated plasma sample.

2. The higher level in serum was dependent on the occurrence of at least the early phase of blood clotting.

3. Hexuronate-containing polysaccharides were recovered from human thrombocytes in the present study and from human leukocytes in studies previously reported.

4. Circulating thrombocytes and leukocytes trapped in the early phase of blood clotting may have been a source of the increased amounts of acid mucopolysaccharides observed in serum as compared to plasma.

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GLUTAMINE ESTIMATION IN CEREBROSPINAL FLUID IN CASES OF LIVER CIRRHOSIS AND HEPATIC COMA

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CLINICAL and experimental observations suggest that faulty nitrogen metabolism may be an important factor in the genesis of hepatic coma. High-protein diet or certain nitrogenous substances given to patients with liver cirrhosis produce a condition clinically indistinguishable from hepatic coma. Elevated blood ammonia levels have been repeatedly reported in these conditions, but in many patients no correlation with the mental state has been found.[1-10]

Investigation of other metabolic factors has recently included measurements of glutamine in the cerebrospinal fluid (C.S.F.). Walshe[2, 12] has reported raised concentrations of glutamine in the C.S.F. of patients in hepatic coma. Whitehead and Whittaker[11] confirmed these findings and proposed simple chemical and chromatographic methods for the estimation of glutamine in the C.S.F.

This report summarizes our experience with glutamine estimation in cerebrospinal fluid as a biochemical aid in the differential diagnosis of hepatic coma.

MATERIALS AND METHODS

Chromatographic Method.—Cerebrospinal fluid, 0.02 ml., is placed on Whatman No. 1 chromatographic paper (20 by 20 cm. square) along a 3 cm. line. An ascending chromatography of 18 hours' duration is performed in the following solvent: Isopropanol, 75 parts; 1 per cent NaCl in distilled water containing 0.5 per cent urea, 25 parts. The color is developed by immersing the paper in ninhydrin reagent and then warming the wet paper at 60° C. for 20 minutes.

Chemical Method.—The method of Whitehead and Whittaker[11] was used.

Materials.—The case material was divided into 5 groups:

1. Patients with hepatic coma (diagnosis based on clinical history and neurologic syndrome). Hepatic coma is referred to as all alterations in the state of consciousness in patients with severe liver disease whether true coma exists or is only expected.[13]

The neurologic status was graded as 0 (normal), 1 (minor disorder of consciousness and the motor system), 2 (gross disorder of consciousness and the motor system), or 3 (coma).[8]

2. Cases of liver cirrhosis without hepatic coma.

3. Patients with liver pathology other than cirrhosis (e.g., infectious hepatitis, fatty degeneration of liver, etc.). None of these patients had central nervous system symptoms.

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Received for publication May 6, 1958.

4. Patients with coma of nonhepatic origin (e.g., diabetic, uremic, and cerebrovascular accidents).

5. Miscellaneous control cases (e.g., meningitis, poliomyelitis, and routine neurologic cases).

RESULTS

By Chromatographic Method.—Chromatograms of C.S.F. in all the control cases showed only a very faint band of glutamine. In those patients with liver cirrhosis uncomplicated by central nervous system symptoms there was only a slightly more intense band of glutamine. On the other hand, in all cases of hepatic coma, a very prominent and easily identified band was seen.

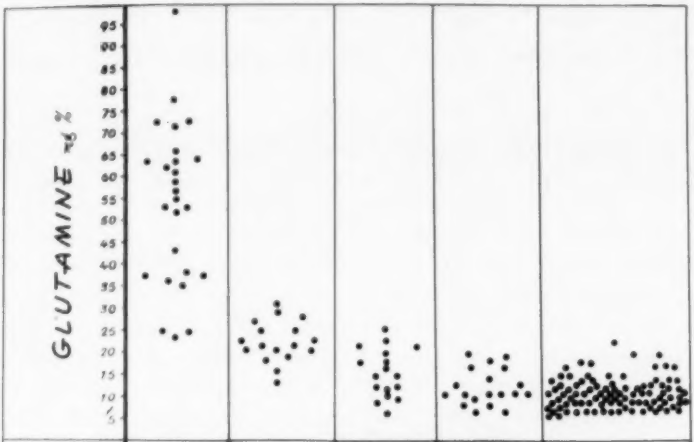
By Chemical Method.—The results of the chemical method are summarized in Fig. 1. The normal levels of glutamine in C.S.F. in our laboratory range from 5 to 22 mg. per cent. Values in patients with cirrhosis of the liver without neurologic manifestations were usually significantly elevated (range 13 to 30 mg. per cent). The highest values were obtained in hepatic coma and there was almost no overlapping with the glutamine levels of the other groups. Two cases demonstrating the value of this test in the differential diagnosis of hepatic coma, and the correlation between the neurologic state and the glutamine level in the C.S.F. are presented.

CASE REPORTS

CASE 1.—A 60-year-old woman has been admitted to the Tel-Hashomer Hospital several times since 1952. Her chief complaints have been weakness, abdominal pain, and recurrent episodes of disturbance in consciousness sometimes to the extent of stupor or even coma (Grade 3). The following combined diagnosis was made: arteriosclerotic heart disease, diabetes mellitus, seropositive syphilis, and cirrhosis of liver (proved by liver biopsy). On Oct. 30, 1957, she was hospitalized again because of "strange behavior" at home. On admission she was apathic and disorientated. Neurologic examination was negative. Her liver and spleen were enlarged. There was no free fluid in the abdomen. An electroencephalogram showed a generalized disturbance characterized by a slowing of the fundamental rhythm to 4 to 6 waves per second. Laboratory findings were: urea 14 mg. per cent, glucose 156 mg. per cent, thymol flocculation strongly positive, and thymol turbidity 9 units. On routine examination of cerebrospinal fluid there were no pathologic findings. Glutamine content of the C.S.F. was 52 mg. per cent.

The patient was put on a low-protein diet and given oral antibiotics. In the course of 5 days her condition improved, and she became alert. The glutamine level in C.S.F. dropped to 21 mg. per cent. Administration of ammonium chloride was followed by a marked deterioration and a rise in the glutamine level. The changes in her condition are summarized in Fig. 2. Handwriting has been used as a test of the mental state and good coordination.[14] A close correlation has been found between the handwriting, the neurologic status, and the glutamine levels in C.S.F. (Fig. 2).

CASE 2.—A 62-year-old man was found unconscious in the street. On admission we found coma, stertorous breathing, spasticity of the lower extremities with bilateral flexor plantar response, but positive ankle clonus. No physical signs suggestive of liver disease were observed. He was not accompanied by relatives and therefore no history whatsoever could be obtained. Laboratory examinations revealed the following: blood urea 38 mg. per cent, blood glucose 137 mg. per cent, no barbiturates found in urine. The glutamine level in C.S.F. was 51 mg. per cent and on the following day 69 mg. per cent. On the



	HEPATIC COMA	LIVER CIRRHOSIS	OTHER LIVER PATHOLOGY	OTHER COMATOSE STATES	CONTROL CASES
Nº OF CASES	26	17	18	21	98
RANGE (mg %)	23 - 96	13 - 30	6 - 24	6 - 19	5 - 22
MEAN ± ST. DEV.	52 ± 17 mg %	21.3 ± 4.2 mg %	15 ± 5.2 mg %	11.4 ± 3.7 mg %	10.3 ± 3.5 mg %

Fig. 1.—Results of the chemical method of estimation of glutamine levels in cerebrospinal fluid.

DATE	TREATMENT	AMMONIUM CHLORIDE	NEUROL. STATUS	HANDWRITING	GLUTAMINE IN C.S.F.
31.10. 1957	ACHROMYCIN LOW PROTEIN DIET		3		52 mg %
1.11	"		3		
3.11	"		2		
7.11	"		0		21 mg %
11.11	"		0		
16.11	"	6 gm	0		
18.11	"	20 gm	3		35 mg %
19.11	"		0		20 mg %

Fig. 2.—Treatment, neurologic status, handwriting, and glutamine levels in cerebrospinal fluid of a patient in hepatic coma (Case 1).

basis of these laboratory results a diagnosis of hepatic coma was made. The patient died 2 days after the admission. Postmortem examination revealed liver cirrhosis and gastrointestinal bleeding.

From the study of 19 cases the following observations concerning the correlation between the glutamine level and the neurologic status were made:

1. In the same patient improvement in the neurologic status was accompanied by a drop in the glutamine level, which rose again on deterioration.

2. No fixed correlation however was found between the grade of coma and the glutamine level in various patients. For instance, in one case with a Grade 3 coma, C.S.F. glutamine of 41 mg. per cent was found, while in another case with a glutamine of 59 mg. per cent only a mild neurologic disturbance (Grade 1) was observed. (Fig. 3.)

CASE	NEUROLOGICAL STATE	GLUTAMINE LEVEL
№ 3	2 3 1 3	64 74 30 69
№ 4	3 1 0 2	41 30 20 34
№ 5	1 3	59 70

Fig. 3.—Correlation between glutamine levels in cerebrospinal fluid and the neurologic state of patients in hepatic coma.

DISCUSSION

The chromatographic method of glutamine demonstration in C.S.F. as described by Whitehead and Whittaker[11] necessitates previous desalting and deproteinization of the fluid. With the method described here, no such preparation is required. This method is well suited for demonstration and identification purposes, but owing to the length of time required for its completion, its value for diagnostic purposes is limited. The chemical method of Whitehead and Whittaker is preferable for this purpose; it requires not more than half an hour for its completion, it is simple, does not require any special reagents or equipment, and every technician versed in blood urea examination can perform it easily.

The results fully confirm the earlier observations that the glutamine level in the C.S.F. is significantly elevated during hepatic coma and that it parallels the neurologic state of the patient. The results suggest that a high glutamine level in the C.S.F. is pathognomonic of hepatic coma. We have never observed

a glutamine level above 35 mg. per cent in a patient without disturbances of consciousness. In comatose states not due to liver pathology, glutamine levels were found to be normal. We know of no other biochemical finding which could, by itself, permit the diagnosis of this condition. A high bilirubin level and the disturbed liver function tests present in advanced liver disease do not exclude the possibility that a patient with such an ailment could be in a state of coma because of a cerebrovascular accident, diabetes, uremia, or barbiturate poisoning. Cases 1 and 2 demonstrate well the value of glutamine estimation in the differential diagnosis of coma in patients suffering from several disorders or in patients whose previous clinical history is unobtainable.

A hypothesis has been proposed that the hepatic coma is caused by interruption of the citric acid cycle by removing α-ketoglutarate and thus blocking the production of the subsequent components of the cycle.[10, 15, 16] On the basis of this hypothesis the elevation of C.S.F. glutamine could be explained as follows: the increased ammonia, reaching the brain in patients with liver disease, combines with the α-ketoglutaric acid to form glutamic acid and subsequently glutamine which may be then found in high concentration in the C.S.F., but not necessarily in the blood because of the blood brain barrier. A logical sequence of such a hypothesis would be the administration to patients in hepatic coma of glutamic acid or α-ketoglutaric acid to bind the excessive ammonia and thus prevent the interruption of the Krebs' cycle in the brain.

The reported failures of the treatment by glutamic acid and α-ketoglutaric acid given orally or intravenously could be explained by the inability of these substances to reach the brain through the above-mentioned barrier. Perhaps administration of glutamic acid or α-ketoglutarate directly into the subarachnoid space would overcome this difficulty.

Of course, these are only speculations as the hypothesis of depletion of α-ketoglutaric acid in the brain in hepatic coma has not been proved, and contradictory evidence has been pointed out by Summerskill and associates[17] who found increased levels of α-ketoglutaric acid in the C.S.F. of patients in hepatic coma. On the other hand, supporting evidence for this theory is afforded by the finding of Bessman[18] that injection of ammonium salts into rats leads to a significant decrease in the concentration of α-ketoglutaric acid in the brain tissue.

SUMMARY

1. Chemical determinations of the cerebrospinal fluid glutamine uniformly revealed an elevation (23 to 96 mg. per cent) in cases of hepatic coma.

2. In cases of liver disease without coma and in cases of coma due to causes other than liver disease, glutamine levels in cerebrospinal fluid were never higher than 35 mg. per cent.

3. The findings suggest that elevation of glutamine in cerebrospinal fluid is pathognomonic of hepatic coma.

4. A simple chromatographic method for the demonstration of glutamine in cerebrospinal fluid is described. It is, however, not suitable for quantitative determination. For rapid laboratory diagnosis the chemical method of Whitehead and Whittaker is preferable.

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URINARY 17-HYDROXYCORTICOSTEROID EXCRETION IN THE NORMAL RHESUS MONKEY

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THE measurement of urinary 17-hydroxycorticosteroid (17-OHCS) concentrations, especially in conjunction with plasma 17-OHCS determinations, has considerable practical value as an index of adrenocorticotrophic (ACTH) secretion. It is particularly suitable for the study of chronic or prolonged alterations in pituitary-adrenal cortical activity over a period of days or weeks, permitting frequent measurements on individual animals without the limiting considerations attendant to repeated venipuncture and depletion of blood elements. The interpretation of urinary 17-OHCS levels must always include the consideration that under certain conditions, such as alterations in renal and hepatic function, or possibly variations in "tissue utilization," the output of the adrenal cortex may not be accurately represented in the urinary output of corticosteroids. The judicious use of plasma 17-OHCS determinations together with urinary measurements, however, often is of considerable value in clarifying the interpretation of results in such instances. Some of the disillusionment occasionally expressed about urinary corticosteroid determinations may be related to the lack of specificity in some of the early methods. In 1953, Glenn and Nelson[1] reported a practicable method for urinary 17-OHCS determination based upon the same general principles as the Nelson-Samuels plasma 17-OHCS method.[2] This method appeared to have the necessary elements for a high degree of specificity, including enzymatic hydrolysis, chloroform extraction, chromatographic separation of corticosteroids from other chloroform-soluble material, and the utilization of the relatively specific Porter-Silber reaction[3] for 17,21-dihydroxy-20-ketosteroids.[3]

The present report is concerned with a systematic evaluation of the precision of this method, the description of some modifications, and an evaluation of the suitability of the method for the study of pituitary-adrenal cortical function in the Rhesus monkey, which we have chosen as the experimental subject for a proposed long-term study of the neural regulation of endocrine secretion.

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From thesis submitted by Nathan R. Rosenthal to the Department of Chemistry, Graduate School, Georgetown University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Received for publication April 10, 1958.

MATERIALS AND METHODS

1. *Biochemical Preparation of Material.*—

Standard hydrocortisone solution: The standard stock solution of hydrocortisone was made from crystals of the free alcohol in a concentration of 100 μg per milliliter in redistilled ethanol. This was stored in brown glass at 4° C.

Penicillin: The commercial antibiotic (Lilly or Pfizer) was dissolved in distilled water to make the concentration 25,000 units per milliliter.

Enzyme: This commercially available solution contains 5,000 units of β-glucuronidase per milliliter. It is obtained from beef liver and is marketed under the trade name Ketodase.*

Buffer: This was made by mixing 114 ml. of 2 M acetic acid with 86 ml. of 2 M sodium acetate resulting in a pH 4.5 buffer.

Other reagents were prepared as described by Glenn and Nelson.[1]

Procedures.—The method of Glenn and Nelson[1] was used except for a modified buffer system and a methanol transfer. Urine samples were either analyzed immediately or preserved in a freezer and were thawed just prior to the start of an analytic run.

Hydrolysis: A 10 ml. aliquot of urine was placed in a 50 ml. glass-stoppered centrifuge tube. The sample was treated with 25,000 units of penicillin G dissolved in 1 ml. of water. Next, 5 ml. of 2 M pH 4.5 acetate buffer was added and finally 10,000 units of the enzyme solution. The tube was stoppered and gently inverted 10 times, after which the cap was removed for a moment to release the gas pressure within. The tube was firmly stoppered and was placed in a 47° ± 0.5° C. bath to incubate for 15 hours (overnight). The tube was cooled to room temperature prior to extraction.

Extraction: To the hydrolyzed mixture 15 ml. of freshly distilled chloroform was added and the tube was vigorously shaken for 30 seconds. Centrifugation for 2 to 3 minutes at 3,500 r.p.m. was sufficient to break the emulsion. The clear chloroform layer was transferred by means of a serum lifter into another centrifuge tube. This extraction procedure was done a total of 3 times, and each successive chloroform layer was added to the first. The pooled chloroform extract was washed twice with 5 ml. portions of 0.1 N NaOH and once with 5 ml. of 0.1 N HCl. The 3 washings were combined and back extracted with 5 ml. of chloroform. To the combined extracts about 3 Gm. of anhydrous sodium sulfate was added and the mixture was shaken until it became water-clear. The dried chloroform was filtered through Schleicher & Schuell filter paper No. 590 into a 125 ml. Erlenmeyer flask; the remaining filter paper and sodium sulfate were washed 3 times with chloroform which was added to the filtrate. The flask was placed in a 47° C. bath, and the solvent was evaporated with a gentle stream of air. The dry residue contained 17-hydroxycorticosteroids and other chloroform-soluble substances. This dried residue was dissolved in 5 ml. of chloroform just prior to chromatography.

Chromatography: The 17-hydroxycorticosteroids were separated from other chloroform extractable substances by passing the mixture through a chromatographic adsorbent. The chromatographic Pyrex column resembles a thistle tube with the dimensions of 13 by 120 mm., constricted to 2 mm. at the lower end and surmounted by a 50 ml. bulb. To prepare the column a small piece of glass wool was placed over the lower constriction and the activated Florisil was packed into the column with the proper "tightness" for a depth of 70 mm. This is a critical step; the "tightness" must be such that the flow of the solvents through the column will require not less than 40 nor more than 70 minutes. Another piece of glass wool was placed atop the packed adsorbent to prevent disturbance during the addition of the sample and the solvents.

The Florisil column was first washed with 15 ml. of chloroform. During the chromatographic step the column must not be allowed to run dry; thus, as the meniscus

*Warner-Chilcott, Laboratory Supply Division, Morris Plains, N. J.

approached the upper end of the adsorbent, the 5 ml. of chloroform containing the sample was quantitatively transferred onto the column. As this meniscus approached the adsorbent, 25 ml. of chloroform was added, followed at the appropriate moment by 25 ml. of 4 per cent methanol in chloroform. All of the eluate collected thus far was discarded. Finally, 45 ml. of 25 per cent methanol in chloroform was added to the column and the eluate collected in a clean 50 ml. Erlenmeyer flask. This fraction contains 17-hydroxycorticosteroid, and at 47° C. was evaporated to dryness under a gentle stream of filtered air.

Colorimetry: The dried steroid residue was dissolved in 5.0 ml. of methanol; 1.0 ml. of this solution was quantitatively transferred to a test tube, and this was evaporated to dryness. The dried residue was dissolved in 1.25 ml. of methanol. Finally, 2.0 ml. of phenylhydrazine-sulfuric acid was added, and the tube was shaken vigorously to assure mixture of the steroid with the coupling agent. The tube was stoppered and incubated at 60° C. for one hour. At the end of this time the tube was cooled, transferred to a 1 cm. light path cuvette, and read against a column blank in a Beckman DU spectrophotometer at 360, 400, and 440 mμ. The column blank was prepared by passing 5 ml. of chloroform through the chromatographic column and treating it in all other subsequent steps in the same fashion as the steroid sample.

Calculations: The absorption factor as described by Allen[5] is the difference between the peak optical density observed at a certain wave length and the average of the optical density readings at two other wave lengths equidistant from the peak. The absorption factor is directly proportional to the concentration of corticosteroid in the sample. According to Allen, absorption factor may be used if the background is linear, or nearly so, with respect to wave length.

In order to confirm that these conditions obtain in the Glenn-Nelson method, the background absorption of monkey urine blanks lacking phenylhydrazine was studied and found to be almost linear within the specified wave length range. A breakdown of the sources of background absorption revealed that the column blank and the urine blank contribute considerably more than the reagent blank to the total background absorption.

2. *Physiologic Samples.*—The collection of 24-hour urine samples from Rhesus monkeys was accomplished with the use of a chair-type restraining apparatus which has been described in detail elsewhere.[6] Briefly, in this chair the monkey can be maintained in the sitting position for as long as several weeks, if necessary. There is virtually complete freedom of the extremities, gentle restraint being maintained only at the neck and waist. A funnel attached to the chair facilitates the collection of urine, and fecal contamination is minimized by means of a removable screen. Preliminary experiments have shown that monkeys adapt readily, within 2 to 4 days, to this situation and thereafter stabilize at blood and urine 17-OHCS levels which are in the same range as those observed when the animals are in their home cages.[7] It was also found that a weekly rhythm exists in levels of animals kept in laboratories, with a depression of 17-OHCS values on weekends, high values on Mondays, but stable values from Tuesday to Friday. The values presented in this report represent only those samples collected between Tuesday and Friday after the animal had been allowed an initial period of 4 days for adapting to the chair.

RESULTS

In order to study the precision and reliability of the analytic method, each of its parts was studied separately in so far as possible.

Chromatography and the Porter-Silber Reaction.—Duplicate samples of cortisol ranging in concentration from 5 to 40 μg were analyzed colorimetrically, one set with, and one set without chromatography through Florisil

columns. The absorption factor means and standard deviations are presented in Table I. A loss of from 5 to 15 per cent occurs when standard quantities of hydrocortisone pass through the Florisil column.

TABLE I. PRECISION OF THE CHROMATOGRAPHIC AND COLORIMETRIC PROCEDURES

CONCENTRATION OF HYDROCORTISONE (μG)	ABSORPTION FACTOR (MEAN ± S.D.*) BEFORE CHROMATOGRAPHY	S.D. (μG)	NO. OF DUPLICATE SETS	ABSORPTION FACTOR (MEAN ± S.D.†) AFTER CHROMATOGRAPHY	S.D. (μG)	NO. OF DUPLICATE SETS
5	0.031 ± 0.004	0.4	4	0.027 ± 0.006	0.6	10
10	0.064 ± 0.004	0.4	4	0.053 ± 0.004	0.4	10
20	0.120 ± 0.004	0.4	4	0.114 ± 0.009	0.9	10
40	0.244 ± 0.014	1.4	4	0.235 ± 0.017	1.7	10

*0.05 probability 7 degrees of freedom t = 2.365.
†0.05 probability 19 degrees of freedom, t = 2.093.

Extraction, Chromatography, and the Porter-Silber Reaction.—The Glenn-Nelson method requires 3 extractions of the urine with 15 ml. of chloroform. To each of six 10 ml. urine samples was added 100 μg of cortisol. Duplicate samples were extracted once, twice, and three times, and the analytic procedure was completed. Three chloroform extractions remove up to 96 per cent of hydrocortisone added to urine, with about 80 per cent of the total steroid accounted for in the first extraction; the second extraction removes about 15 per cent of the steroid, and the third extraction removes only 1 to 2 per cent more. A fourth extraction does not appreciably improve the recovery.

TABLE II. HYDROCORTISONE RECOVERY FROM URINE WITH AND WITHOUT β-GLUCURONIDASE HYDROLYSIS

HYDROCORTISONE (μG) ADDED PER 10 ML.	RECOVERY BEFORE HYDROLYSIS	PER CENT RECOVERED	RECOVERY AFTER HYDROLYSIS	PER CENT RECOVERED
0	0	0	0	0
6.2	6.0	97	6.5	105
12.5	13.0	104	12.5	100
25.0	24.0	96	24	96
37.5	36.0	96	33.5	89
50	42.5	85	47.5	95
62.5	58	93	–	–
75	68	88	–	–
100	90	90	88	88
125	117	93	106	85
150	130	87	129	86
200	182	91	181	90
Mean Recovery		92.7		92.6

Hydrolysis, Extraction, Chromatography, and Colorimetry.—To determine what recovery of corticoid from urine could be expected, 6.2 to 200 μg of hydrocortisone was added to aliquots of urine just prior to hydrolysis. Because hydrolysis may be destructive to the side chain, a similar experiment was done except that neither buffer nor enzyme was added to the samples. The data shown in Table II indicate good recovery of hydrocortisone from

urine and that enzymatic hydrolysis is not destructive to the steroid with a dihydroxyacetone side chain. When duplicate aliquots of urine were examined by the Glenn-Nelson method, omitting both enzyme and buffer in one sample of each pair, it was shown that 10 to 25 per cent of the total 17-OHCS content was excreted unconjugated with glucuronic acid.

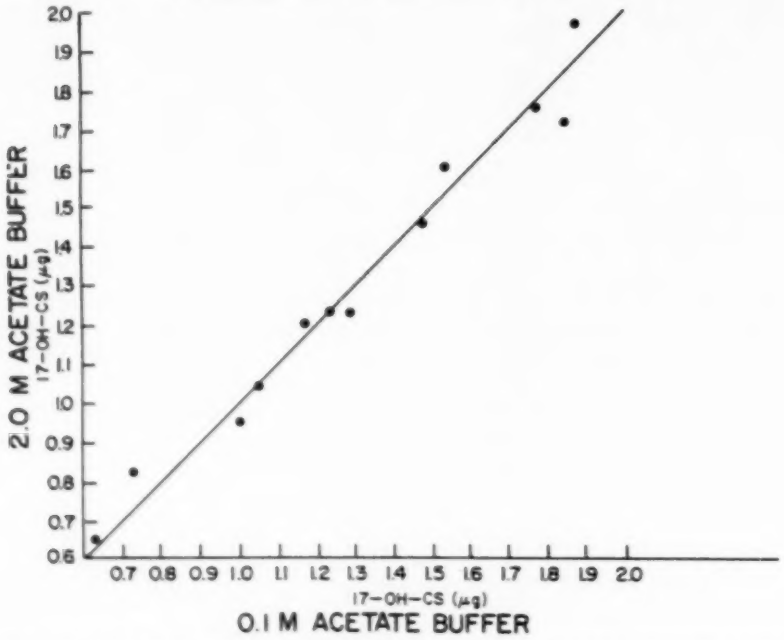


Fig. 1.—Comparison of 17-hydroxycorticosteroid concentration in urine after enzymatic hydrolysis in two different acetate buffer concentrations.

The Buffer.—The hydrolytic procedure of Glenn and Nelson[1] employs the adjustment of the urine to pH 4.5 using 0.1 N acetate and acetic acid by dropwise addition. This step was simplified by the addition of 5 ml. of 2 M pH 4.5 buffer just before hydrolysis was begun. The 17-OHCS values obtained in duplicate samples with the two buffer systems are shown in Fig. 1. The small deviations from the line of equality indicate that the two buffer systems are entirely comparable. Hydrolysis of urine in the presence of the buffer, without β-glucuronidase, liberates from 40 to 75 per cent of the glucuronide conjugated 17-hydroxycorticosteroid.

Stability of 17-Hydroxycorticosteroid in Urine.—Multiple aliquots of 6 monkey urine samples were analyzed at intervals of 3 to 5 weeks to test the duplicability of the method and the effect of freezing on the stability of the steroid. The data indicate good stability of corticosteroids in frozen urine over a period of at least 6 months, even with occasional thawing and refreezing of samples.

Reproducibility of the Complete Method.—The 4 steps of the procedure were tested for precision in reproducing values from day to day. Multiple aliquots of the same urine were analyzed on several days to test the duplicability of the method. The data are presented in Table III and indicate a high degree of precision.

TABLE III. REPRODUCIBILITY OF THE GLENN-NELSON URINARY 17-HYDROXYCORTICOSTEROID METHOD

ANALYTIC DATE	ABSORPTION FACTOR	MICROGRAMS OF 17-OHCS
June 28, 1955	0.059	10.7
	0.062	11.2
	0.065	11.7
	0.064	11.5
June 30, 1955	0.063	11.4
	0.063	11.4
	0.054	9.8
	0.061	11.0
July 19, 1955	0.066	11.9
	0.064	11.5
	0.066	11.9
	0.062	11.2
Mean	0.063	11.4
	±0.004	10.7-11.9

TABLE IV. URINARY 17-HYDROXYCORTICOSTEROID EXCRETION BY NORMAL RHESUS MONKEYS

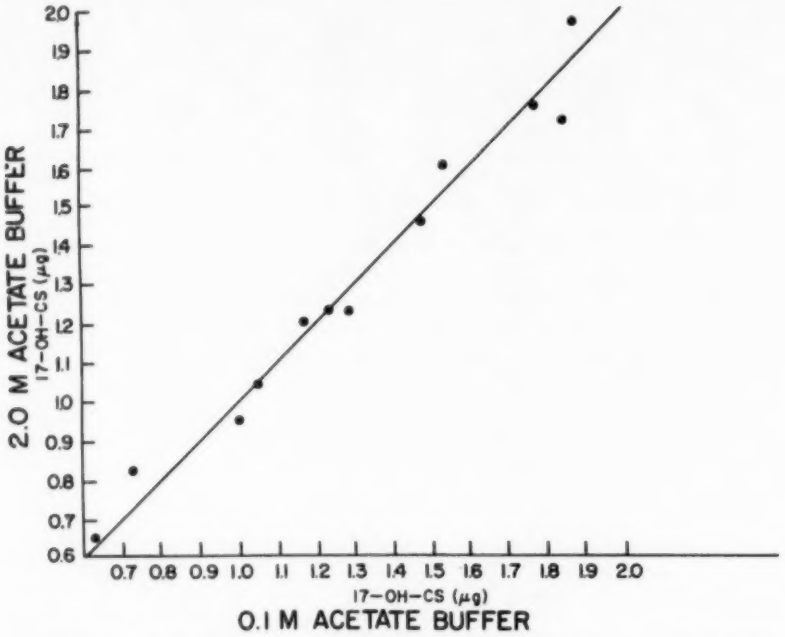
MONKEY	MEAN 17-OHCS EXCRETION (MG./24 HR.)	RANGE (MG./24 HR.)	NUMBER OF OBSERVATIONS ON EACH ANIMAL
17	1.7	1.2-2.3	11
22	1.7	1.1-3.4	8
23	1.2	0.8-1.5	12
26	1.5	0.8-2.4	17
27	1.4	0.7-1.9	10
47	0.9	0.4-1.0	5
37	0.7	0.6-0.9	4
Mean	1.3	0.8-1.9	
			Total 67

Urinary 17-Hydroxycorticosteroid Excretion by the Monkey.—The total 24-hour urinary 17-OHCS excretion of 7 normal monkeys was measured between Tuesday and Friday in a total of more than 60 instances after the animals were adapted to the experimental chair. These data are presented in Table IV and indicate that each animal has a base-line level with a fairly narrow range of day-to-day variation. In fact, the variation from animal to animal, kept in a similar environment, is quite small. Slightly higher mean 17-OHCS excretion values in the first 5 monkeys probably reflect the fact that their experimental chairs were located in a chemistry laboratory where there was moderate, although stereotyped, daily activity; whereas lower values are seen in the latter pair of animals which were isolated in a room where there was considerably less daily activity.

DISCUSSION

The data presented indicate that the analytic method of Glenn and Nelson has a high degree of precision and dependability. The standard deviation

urine and that enzymatic hydrolysis is not destructive to the steroid with a dihydroxyacetone side chain. When duplicate aliquots of urine were examined by the Glenn-Nelson method, omitting both enzyme and buffer in one sample of each pair, it was shown that 10 to 25 per cent of the total 17-OHCS content was excreted unconjugated with glucuronic acid.



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DISCUSSION

The data presented indicate that the analytic method of Glenn and Nelson has a high degree of precision and dependability. The standard deviation

at the various concentrations of cortisol approaches a constant of about 0.6 μg between 5 and 30 μg for the combined chromatographic and colorimetric steps. In the final calculation of the total 24-hour urinary 17-OHCS excretion expressed in milligrams this deviation does not exceed 0.2 mg. The standard deviation of the 40 μg concentration of hydrocortisone is more than twice that of the lower concentrations, because the optical density at 400 mμ is so great that it must be measured in the region of the Beckman scale where sensitivity is low. The duplicability of the method was found to be excellent, with replicate analyses of urine samples from day to day invariably falling within the standard deviation limits described above. The stability of 17-hydroxycorticosteroids in frozen urine samples was constant for periods at least up to 6 months, even with occasional thawing and refreezing.

Because the background absorption due to nonsteroidal material is nearly linear with respect to wave length, it is possible to use the absorption factor for calculation after the method of Allen.[5] The absolute absorption factors vary but little from day to day, making practicable the use of a standard curve of 17-OHCS concentration. The relationship between the absorption factor and concentration of 17-hydroxycorticosteroid is linear and obeys Beer's law within the specified concentration range.

When standard amounts of cortisol were added to duplicate samples of urine, only one of which was hydrolyzed with the enzyme and the buffer while the other was kept refrigerated, recovery of 90 per cent of the added steroid was obtained from both samples. This indicates that the hydrolytic procedure is nondestructive to cortisol.

The buffer system employed in the enzymatic hydrolysis of the glucuronide-conjugated 17-hydroxycorticosteroid was simplified by the use of a 2 M buffer which, due to its high concentration, eliminated the necessity of checking the pH of individual samples. An experimental comparison of the 2 M buffer with the 0.1 M buffer established that the difference in ionic strength did not interfere with the action of the enzyme. A finding of some incidental interest was that the buffer alone had some hydrolytic effect ranging from 40 to 75 per cent as much as that obtained with the enzyme. A significant though small fraction (up to 15 per cent) of the total 24-hour 17-hydroxycorticosteroid is excreted apparently in the free state, as determined in urine which was not hydrolyzed with either buffer or enzyme.

In general, it appears that this analytic method is an extremely reliable tool for the determination of urinary 17-OHCS concentrations. The preparation of materials must be rigidly uniform, particularly the preparation of the Florisil.

This method appears to be well suited for the study of adrenal cortical function in the Rhesus monkey, particularly since Bush[8] has observed cortisol to be the principal secretory product of the adrenal cortex in the monkey. Also, recent preliminary experiments in our laboratory with intravenous administration of large amounts of crystalline cortisol (6 mg. per kilogram)

in the monkey indicate that approximately 20 per cent of the injected hormone is recovered in the urine as 17-hydroxycorticosteroids.[9] It is of interest that the monkey apparently excretes about 5 to 8 times as much 17-hydroxycorticosteroid, on the basis of body weight, as do human beings.

The experimental chair appears to be a very suitable apparatus for urine collection in monkeys. There is no urine loss from projection of the urine stream, due to the limited mobility of the animal. Twenty-four-hour urine volumes usually range between 200 and 300 ml. in normal adult Rhesus monkeys on an average diet with fluid ad libitum. As might be expected, some monkeys show moderate elevations in 17-OHCS excretion for the first few days after being placed in the chair apparatus.[7] After about the fourth day, however, the levels become remarkably stable and remain so for many weeks. It is well, perhaps, to call attention to some observations which indicate that levels of adrenal cortical activity in the monkey quite sensitively reflect changes in the surrounding environment. Animals kept in chairs in a busy, active laboratory apparently run higher base-line levels than animals in separate stalls in a private room where there is little noise or activity. This finding suggests that one must define "normal" levels in terms of the particular conditions in a given laboratory and that it is preferable to do longitudinal studies in individual animals kept in a constant environment whenever possible. The present data indicate that base-line levels from animal to animal do not vary greatly when the animals are kept under constant conditions. Recent experiments have shown very substantial elevations (two- to four-fold increases) in urinary 17-OHCS levels in monkeys receiving such agents as ACTH or reserpine, indicating that this measurement may be a sensitive indicator of adrenal cortical activity in the monkey.[9]

SUMMARY

The Glenn-Nelson analytic method is a reliable tool for the measurement of 17-hydroxycorticosteroid concentration in urine, as shown by a statistical analysis of precision. The precision of the method at the 5 μg concentration is ± 0.6 μg. The standard deviation remains nearly constant up to 30 μg. Multiple aliquots of the same monkey urine repeatedly analyzed at monthly intervals give reproducible results within this range. The conditions employed for the hydrolysis of urinary glucuronide-conjugated 17-hydroxycorticosteroids are nondestructive to the steroid molecule.

Normal Rhesus monkeys showed mean 24-hour 17-hydroxycorticosteroid excretion values from 0.7 to 1.7 mg. There is relatively little variation in day-to-day levels in normal monkeys after adaptation to the experimental chair, providing that the environment is reasonably constant and stereotyped. Some aspects of the environmental influences on adrenal cortical activity are discussed.

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THE INFLUENCE OF 17-HYDROXYCORTICOSTERONE ON VIRAL HEPATITIS OF MICE

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RECENT reports have suggested that adrenocorticotrophic hormone and corticosteroids beneficially influence the course of human hepatitis in that these agents promote a sense of well-being, increase the appetite, and cause an increased rate of diminution of serum hyperbilirubin.[1, 2] In spite of these favorable effects of corticosteroids, the laboratory criteria of liver disease (sulfobromophthalein excretion, thymol turbidity, cephalin cholesterol flocculation, serum alkaline phosphatase, serum total cholesterol, and cholesterol esters) remain abnormal for approximately the same length of time as is observed in patients with hepatitis not treated with corticosteroids.[3] Liver biopsy studies have demonstrated the accelerated disappearance of the inflammatory response but no significant change in the extent of hepatic tissue necrosis.[2]

Viral hepatitis of mice in some aspects is similar to hepatitis of man.[4] Using this experimentally transmitted form of viral hepatitis in mice, it was decided to study the influence of 17-hydroxycorticosterone (hydrocortisone) on viral hepatitis. Previous studies of viral hepatitis in mice have indicated the relationship of virulence of virus, size of virus inoculation, and histologic evidence of hepatic necrosis to serum transaminase activity.[5] Accordingly, it was planned to evaluate the effects of hydrocortisone on viral hepatitis of mice by following the serial plasma and tissue activities of glutamic oxalacetic transaminase (GOT),[6] glutamic pyruvic transaminase (GPT),[7] as well as lactic dehydrogenase (LD),[8] and glutathione reductase (GR).[9] The histologic appearance of liver tissue was to be used as a measure of the extent of tissue necrosis.

MATERIALS AND METHODS

The mice used in these studies were F, hybrids of C-58 X BALB (F_1) cross, weighing from 16 to 19 grams. They were fed Purina mouse food and allowed water ad libitum.

In each study, mice were divided into 4 groups. Group 1 consisted of untreated normal mice. Group 2 received hydrocortisone daily, injected subcutaneously, in the doses noted in the text. The mice in Group 3 were inoculated with 0.1 ml. of 10^{-3} dilution of a filtrate of mouse hepatitis virus. The group 4 mice were inoculated with the same dilution of the hepatitis virus and received daily subcutaneous doses of hydrocortisone. Groups 1 and 3 were the controls of Groups 2 and 4, respectively.

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Supported in part by Grant C 4251 from the National Cancer Institute.
Received for publication Aug. 29, 1958.

Three mice from each of the 4 groups were sacrificed at 2- or 3-day intervals. They were bled from the brachial artery with a heparinized syringe and the bloods of the 3 mice from each group were pooled in a test tube. A small fragment of liver was excised from each mouse for histologic examination after which the remaining mouse livers of each group were pooled. They were homogenized with a Teflon pestle homogenizer and made up to 10 per cent by weight in 10 per cent dextrose solution. Four different enzyme activities were studied in plasma and hepatic tissue homogenated, namely, GOT, GPT, LD, and GR. The methods for enzyme activity determinations have been described elsewhere.[6-9]

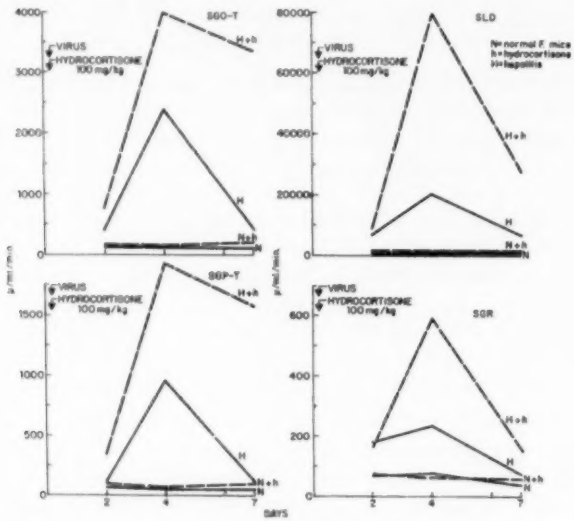

Fig. 1.—The evolution of 4 different plasma enzymes in 4 groups of mice: normal controls (N), controls receiving hydrocortisone 100 mg./Kg. daily (N+h), mice with viral hepatitis (H), and mice with viral hepatitis receiving hydrocortisone 100 mg./Kg. daily (H+h).

RESULTS

Fig. 1 shows the variations of serum enzymes in normal untreated mice, normal mice receiving 100 mg. hydrocortisone per kilogram of body weight, in mice with hepatitis, and in mice with hepatitis receiving hydrocortisone in the same amount as the uninjected control animals. Whereas there is no significant difference in the plasma enzyme activities of noninfected mice, whether receiving hydrocortisone or not, all 4 plasma enzyme activities are appreciably more elevated in the infected animals receiving hydrocortisone than in those mice with hepatitis but not receiving hydrocortisone. It is evident that while on the seventh day the infected mice receiving no hydrocortisone show a tendency for the serum enzyme activities to return to normal values, the hepatitis mice receiving hydrocortisone continue to have increased activities of the 4 serum enzymes measured.

A second colony of animals received no hydrocortisone until the fourth day following inoculation with hepatitis virus and then the hormone was given in the amount of 100 mg. per kilogram daily; the data from this experiment are

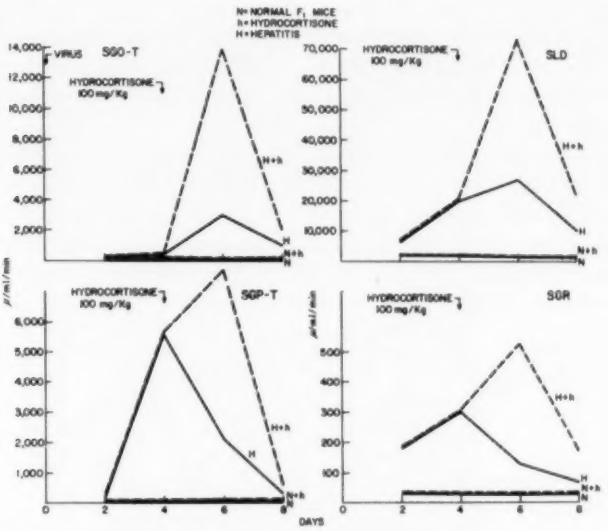

Fig. 2.—The evolution of 4 different plasma enzymes in 4 groups of mice: normal controls (N), controls receiving hydrocortisone 100 mg./Kg. daily since the fourth day (N+h), mice with viral hepatitis (H), and mice with viral hepatitis receiving hydrocortisone 100 mg./Kg. daily after the fourth day (H+h).

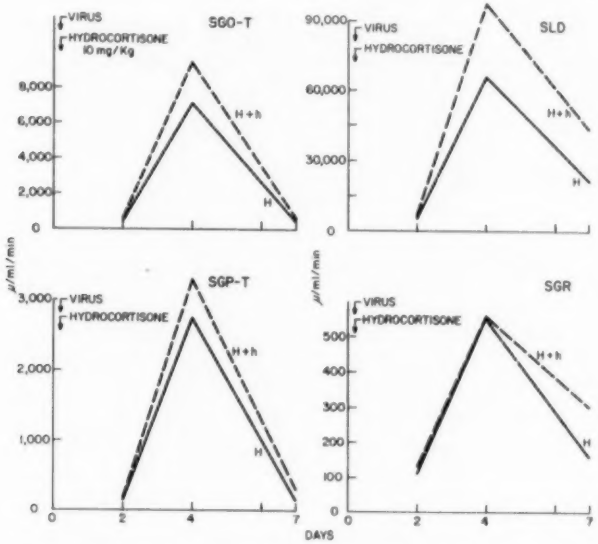

Fig. 3.—The evolution of 4 different plasma enzymes in 2 groups of mice, one with viral hepatitis (H) and the other also with viral hepatitis but treated with 10 mg./Kg. of hydrocortisone daily (H+h).

presented in Fig. 2. A definite rise in serum enzyme activities is evident on the sixth day following 2 subcutaneous doses of hydrocortisone, although at the eighth day the plasma transaminase activities tend to return to normal values; lactic rehydrogenase and glutathione reductase activities continue at abnormal levels.

Fig. 3 presents the data from the study of a colony of mice which received only 10 mg. per kilogram of hydrocortisone daily. In this experiment 2 groups of mice were studied. One group was inoculated with hepatitis virus while another group with hepatitis virus received hydrocortisone. The hepatitis mice receiving the hydrocortisone show higher plasma activities than the hepatitis control mice which did not receive adrenocorticosteroid.

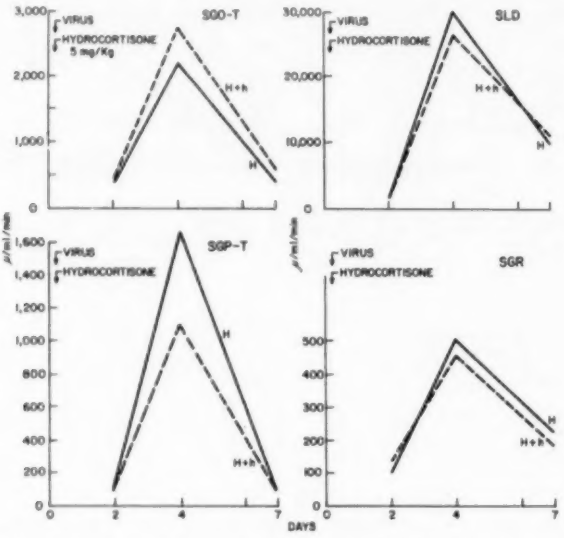

Fig. 4.—The evolution of 4 different plasma enzymes in 2 groups of mice, one with viral hepatitis (H) and the other also with viral hepatitis but treated with 5 mg./Kg. of hydrocortisone daily.

A final group of mice with hepatitis received the hormone in the amount of 5 mg. per kilogram and the observations are compared with those from a similar group which received no hydrocortisone (Fig. 4.) The differences in plasma enzyme activities are not significant.

The enzyme activities of the liver homogenates of the different groups of mice was estimated. No significant difference of enzyme activities measured were noted in the homogenized tissues of those animals which received hydrocortisone and of those which did not. Although not consistently, the histologic appearance of the livers of the injected mice which received hydrocortisone showed more areas of focal necrosis than the livers of the normal and injected mice which did not receive the hormone (Figs. 5 to 8).

Fig. 5.

Fig. 6.

Fig. 5.—Histologic section of normal mouse liver.
Fig. 6.—Histologic section of normal mouse liver after being injected with 100 mg./Kg. of hydrocortisone subcutaneously for two consecutive days. Notice the abundant glycogen deposits in the liver cells.

Fig. 7.

Fig. 8.

Fig. 7.—Histologic section of mouse liver on the sixth day after inoculation of hepatitis virus.
Fig. 8.—Histologic section of mouse liver on the sixth day after inoculation of hepatitis virus and after receiving 100 mg./Kg. of hydrocortisone subcutaneously on the fourth and fifth days postinoculation. Notice the larger extent of necrosis of the hepatic parenchyma as compared to Fig. 7.

DISCUSSION

Although it has been reported that in the livers of normal rats, transaminase activities are stimulated by glucocorticoids,[10-13] the same effect in plasma or liver tissue of the mice without hepatitis could not be demonstrated. In the case of mice with hepatitis however, a significant difference was found in the amount of the enzymes in the plasma, but not in the liver homogenates. Moreover, the activity of the 4 enzymes studied was higher in the mice with hepatitis which received hydrocortisone, than in the control hepatitis mice. This significant difference is especially remarkable when the disease is at its peak, between the fourth and the sixth days, and decreases when the animals spontaneously recover from the viral infection.

Other workers have reported an increase in the virulence of virus diseases in cortisone-treated animals.[14-19] The observations herein reported suggest another instance of an adverse, although more subtle, effect of hydrocortisone in mouse viral hepatitis. This is reflected by the increased levels of enzyme activity in the hydrocortisone-treated mice as compared with the controls. This effect is more apparent when large doses of the adrenocorticosteroids were given.

SUMMARY

1. An attempt was made to detect whether treatment with hydrocortisone affected the plasma levels of 4 different enzyme systems in normal mice and in mice with hepatitis.

2. The 4 enzymes studied were: glutamic oxalacetic transaminase, glutamic pyruvic transaminase, lactic dehydrogenase, and glutathione reductase. There was no difference observed in the plasma levels of the enzymes in the normal mice receiving hydrocortisone. However, a significant increase was noted in the hydrocortisone-treated mice with hepatitis.

3. Using serum enzyme activities as an index of severity of mouse viral hepatitis, it appears that hydrocortisone may exert an adverse effect on the natural course of the experimentally induced infection.

We are indebted to Miss Joan Pasternak and Mr. Roger Tartar for their technical assistance.

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THE CONVERSION OF CORTISONE TO 17-KETOSTEROIDS IN PATIENTS WITH CARCINOMA OF THE PROSTATE AND OF THE LUNG

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THE administration of large doses of cortisone has been shown to produce a marked increase in the urinary 17-ketosteroid excretion of patients with carcinoma of the prostate.[13] When 300 mg. of cortisone acetate was given daily to these patients, the average increment in 17-ketosteroid excretion over the base-line period of no therapy was 21.8 mg. per 24 hours. In contrast, control subjects with various illnesses treated similarly showed an increase of only 4.3 mg. per 24 hours. Subsequent studies have confirmed this finding, demonstrated a similar effect of cortisol, and indicated that this phenomenon is not specific for carcinoma of the prostate, although this is the only disease so far in which it has been observed regularly.[14] Other investigators have also observed a low conversion of cortisone (or cortisol) to 17-ketosteroids in normal subjects,[2, 4, 12] in patients with rheumatoid arthritis,[8, 10] and in an adrenalectomized unilaterally orchiectomized patient,[7] while an increased conversion was observed in patients with carcinoma of the prostate.[6, 15] The latter was also demonstrated in an experiment with tracer doses of C^{14}-labeled cortisol.[9] Bonner and associates[2] were unable to confirm this effect initially but have since observed it in 5 out of 6 patients with carcinoma of the prostate.[3]

This study was undertaken to identify the compounds represented among the excess urinary 17-ketosteroids of patients with carcinoma of the prostate given cortisone, and to determine whether the metabolism of cortisone in these patients differed from that in normal subjects. For comparison, studies were also performed in 3 cases of carcinoma of the lung, a disease in which major increases in 17-ketosteroid excretion are only occasionally observed during cortisone administration.[14]

METHODS

Patients with carcinoma of the lung and prostate were taken consecutively without selection. The clinical data relative to these subjects are presented in Table I. The experimental period was divided into 3 sections: 3 days of no steroids; 3 days of 30 mg. of oral prednisone daily; and 3 days of 30 mg. prednisone plus 300 mg. cortisone acetate daily, administered orally. Complete 24-hour urine specimens were collected in bottles

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Aided by a grant from The American Cancer Society.
Received for publication Sept. 9, 1958.

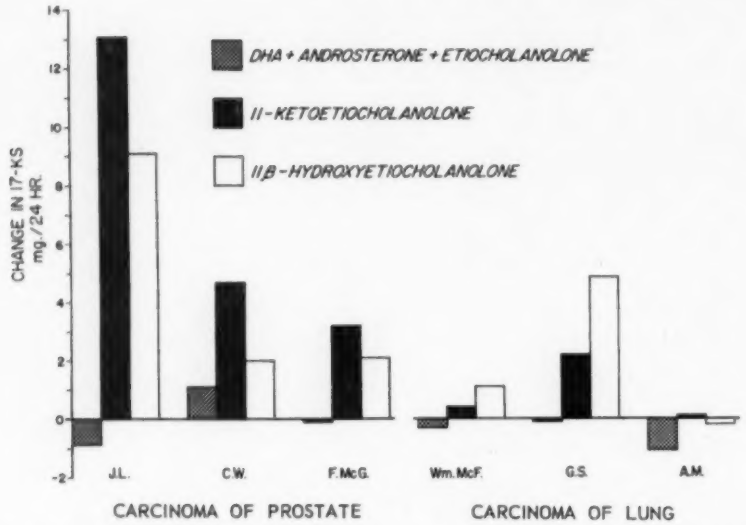

Fig. 1.—The change in urinary 17-ketosteroid excretion during the cortisone period as compared to the base line or control periods.

TABLE I. CLINICAL DATA

PATIENT (ALL MEN)	AGE	DIAGNOSIS	RELEVANT CLINICAL AND LABORATORY DATA
C. W.	71	Anaplastic carcinoma of prostate	Orchiectomy and institution of estrogen therapy 8 mo. previously. Osteolytic metastases of pubic bone, ischium, and humerus. Acid and alkaline phosphatases slightly elevated. Bone pain
F. McG.	65	Adenocarcinoma of prostate	Large prostatic mass, bulging into the rectum, 10 mo. previously. Orchiectomy and institution of estrogen therapy, 9 mo. previously. Osteoblastic metastases of pelvis and ribs. Acid and alkaline phosphatases normal. Essentially asymptomatic
J. L.	55	Adenocarcinoma of prostate	Clinical onset 7 mo. previously. Diagnosis established immediately before study, and therapy not yet instituted. Large prostatic tumor; osteolytic and osteoblastic metastases of pubic rami. Acid phosphatase, upper limit of normal; alkaline phosphatase elevated. Bone pain
A. M.	64	Anaplastic carcinoma of lung	Cervical node metastases, widespread osteolytic metastases, rapid downhill course
G. S.	73	Anaplastic carcinoma of lung	Cervical node metastases
W. McF.	73	Carcinoma of lung	Paratracheal and hilar metastases; superior vena cava syndrome

containing 10 ml. of 6 N hydrochloric acid, and refrigerated until the completion of the experiment. The first urine collections of the prednisone and prednisone-cortisone periods were discarded, since previous experience had shown that at least 24 hours of steroid administration is necessary before an effect on 17-ketosteroids is evident.[13]

Urines were hydrolyzed with acid, extracted, purified, and analyzed by a previously described method.[11] When necessary, two or more extracts were pooled to give a total 17-ketosteroid content of 5 to 15 mg. One extract from each period (when possible) was subjected to gradient elution chromatography. The eluted 17-ketosteroids were measured by the Zimmerman reaction and identified by infrared spectroscopy.[11] The androsterone and etiocholanolone fractions were also analyzed by the method of Bitman and associates[1] for the content of $\Delta^{9,11}$-unsaturated analogues, the artifacts produced by acid hydrolysis from the corresponding 11(β)-hydroxy compounds.

Identification by infrared spectroscopy was sometimes difficult, particularly in those cases in which the extract represented 2 to 4 days' collection of urine. With such large ratios of extraneous material to 17-ketosteroids, our method of purification was inadequate. However, identification of the 17-ketosteroids was satisfactory in all urines collected during administration of cortisone acetate.

RESULTS AND DISCUSSION

The results are shown in detail in Table II and summarized in Fig. 1. The control levels of 17-ketosteroids were lower than those usually seen for several reasons. All subjects were elderly men, and 2 of the patients had been castrated. In addition, the Allen method of correction was employed, which tends to give lower values, especially in the case of extracts already having low titers.[5] Also, each steroid is expressed in terms of itself rather than dehydroepiandrosterone (DHA). Since most of the steroids are more chromogenic than DHA, a decrease in values results.

Prednisone was given in order to depress adrenal function before administration of cortisone acetate. It was felt that this would give a more reliable base line than the period during which no steroid was given. Unfortunately, 17-ketosteroid excretion during these first two periods was so low in many cases that the extracts from the two periods had to be combined. In 2 cases prednisone did produce a slight depression in the identified 17-ketosteroids with respect to the values in the control periods.

All 3 patients with carcinoma of the prostate showed an unequivocal increase in 11-oxy-17-ketosteroids during the cortisone period. In all cases the indicated conversion of cortisone to 17-ketosteroids exceeded that normally observed.[4] It is probably significant that the most pronounced increase was in Patient J. L. who had had no therapy. Previous serial studies of total 17-ketosteroid excretion during cortisone administration indicated that the characteristic rise in patients with carcinoma of the prostate was often diminished after orchiectomy and the institution of estrogen therapy.[14]

In all 3 patients with carcinoma of the prostrate, the rise in 11-ketoetiocholanolone exceeded that in 11(β)-hydroxyetiocholanolone. The single patient with carcinoma of the lung who showed a considerable rise in 17-ketosteroids, on the other hand, had a 1:2 ratio of 11-ketoetiocholanolone to 11(β)-hydroxyetiocholanolone. The other 2 patients with carcinoma of the

TABLE II. URINARY 17-KETOSTEROID EXCRETION (MG. PER 24 HR.) DURING A CONTROL PERIOD, DURING PREDNISONE ADMINISTRATION (30 MG. PER DAY), AND DURING THE ADMINISTRATION OF PREDNISONE AND CORTISONE ACETATE (300 MG. PER DAY)

PATIENT	PERIOD	DEHYDROEPI-ANDROSTER-ONE	ANDROS-TERONE	11(β)-HY-DROXYAN-DROSTERONE*	ETIOCHO-LANOLONE	11-KETOETIO-CHOLANOLONE	11(β)-HYDROXY-ETIOCHOLANO-LONE†	TOTAL
Carcinoma of prostate								
J. L.	Control	1.6	1.2	0.6	0.2	0.6	0.2	4.4
	Prednisone	0.6	1.1	0.8	0.5	0.5	0.1	3.6
	Prednisone and cortisone	0.6	0.3	0.4	0.4	13.6	9.2 (8.3)	24.5
C. W.	Control and prednisone	0.0	0.1	0.0	0.3	0.0	0.4 (0.4)	0.8
	Prednisone and cortisone	0.6	0.8	1.1	0.1	4.7	2.4 (1.9)	9.7
F. McG.	Control	0.3	0.4	0.0	0.5	0.1	0.4 (0.2)	1.7
	Prednisone and cortisone	0.4	0.5	0.1	0.2	3.3	2.5 (1.5)	7.0
Carcinoma of lung								
Wm. McF.	Control	0.5	0.5	0.4	0.7	0.2	0.0	2.3
	Prednisone	0.4	0.4	0.2	0.7	0.2	0.1	2.0
	Prednisone and cortisone	0.4	0.3	0.1	0.5	0.6	1.2 (0.9)	3.1
G. S.	Control and prednisone	0.2	0.2	0.1	0.6	0.1	0.1	1.3
	Prednisone and cortisone	0.3	0.4	0.4	0.4	2.3	5.0 (4.3)	8.8
A. M.	Control and prednisone	0.5	0.3	0.4	1.0	0.1	0.9 (0.6)	3.2
	Prednisone and cortisone	0.2	0.2	0.1	0.3	0.2	0.7 (0.2)	1.7

*Measured as the hydrolytic artifact, $\Delta^{9,11}$-androsten-3(α)-ol-17-one.

†The sum of 11(β)-hydroxyetiocholanolone and its hydrolytic artifact, $\Delta^{9,11}$-etiocholen-3(α)-ol-17-one, the latter being shown in parentheses.

lung showed only small changes in 17-ketosteroid excretion during the cortisone period. This is in keeping with our previous experience, where 1 out of 6 patients with pulmonary carcinoma showed a rise in total 17-ketosteroids comparable to that regularly seen in prostatic cancer.[14]

The compounds principally responsible for the increased 17-ketosteroid excretion observed in this study, 11-ketoetiocholanolone and 11(β)-hydroxyetiocholanolone, are normal metabolites of cortisone. Our studies do not, therefore, indicate any major qualitative departure from the normal metabolism of cortisone in carcinoma of the prostate.

The unusual pattern of cortisone metabolites in carcinoma of the prostate may represent steroid metabolism by the cancer or it may be due to secondary host changes, with possible alterations of liver function in these patients. In order to shed additional light on this question, we have initiated studies in which slices of prostatic carcinoma are incubated with C^{14}-labeled cortisone. Our initial experiments of this type indicate that prostatic carcinoma slices can metabolize cortisone in vitro. Thus, the abnormal urinary steroid pattern after cortisone admininstration in patients with prostatic carcinoma may be due, at least in part, to steroid metabolism by this tumor.

SUMMARY

All 3 patients with carcinoma of the prostate and 1 of 3 patients with carcinoma of the lung converted cortisone to 11-ketoetiocholanolone and 11(β)-hydroxyetiocholanolone in amounts greater than normal. In all of the patients with carcinoma of the prostate, 11-ketoetiocholanolone was the predominant 17-ketosteroid metabolite of cortisone, in contrast to the patients with pulmonary cancer where 11-hydroxyetiocholanolone predominated. No unusual metabolite of cortisone was identified in the urine of these patients.

We are indebted to Mr. Lavalle Neely for the infrared analyses.

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EFFECT OF CHLORAMPHENICOL ON QUALITATIVE ASPECTS OF ANTIBODY PRODUCTION AGAINST ENTEROBACTERIACEAE

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ONE of the interesting features regarding chloramphenicol therapy in enteric fever has been the finding that inadequate treatment may lead to a considerable increase in the incidence of relapse as compared with nontreated cases or those adequately treated. In particular, it appears that the high relapse rates under these circumstances are related to the duration of treatment rather than to total dosage of antibiotic. It has usually been suggested that this results from a failure of antibody production due to inadequate antigenic stimulus, resulting from the bacteriostatic effect of the antibiotic. In a previous publication, however, we have shown that clinical relapse usually takes place at a stage of illness when antibody levels may be very high.[1] In view of this observation it was suggested that failure of humoral response is not the main feature in bringing about the high relapse rate, but rather that the antibiotic may in some way bring about an increase in the numbers of tissue forms of the organisms, perhaps by way of increased phagocytosis, thus creating a potential reservoir of organisms available for reinvasion of the blood stream.

A further possible explanation, however, presents itself, namely, that chloramphenicol may in some way affect the qualitative aspects of antibody so as to lead to a decrease in its protective power. Some observations regarding the qualitative nature of antibody under these circumstances are presented below.

MATERIALS AND METHODS

Antigens.—The following strains were used for the immunization of rabbits: (1) *Salmonella typhosa* (strain 0.901, N.C.T.C. 5759); (2) *Escherichia coli* (strain 5396/38—a potent source of Vi antigen); and (3) *S. typhimurium* (freshly isolated strain).

Organisms were cultured daily in nutrient digest broth,* from an initially smooth colony. Suspensions for immunization were then prepared by subculturing onto nutrient agar slopes, harvesting the growth (after 18 hours' incubation at 37° C. in sterile 0.85 per cent saline), and standardizing to a concentration of 10^6 organisms per milliliter by means of plate counts. Healthy adult rabbits, weighing approximately 3,500 grams, were injected intravenously starting with a dose of 0.2 ml. on the first day, followed by a replicate dose on the third, fifth, and seventh days and followed by two further doses of 0.4 ml. on the eleventh and fourteenth days.

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Received for publication Sept. 10, 1958.

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Antibiotic Treatment.—Fifty per cent of the animals in each group, chosen at random, were given chloramphenicol by daily intramuscular injection in a dose of 12.5 mg. per kilogram of body weight. The first injection was given on the same day as the first immunizing injection, and antibiotic therapy was continued for 3 days following the last immunizing injection—a total of 17 days' treatment.

Bleeding of Animals.—Rabbits were bled into sterile tubes from the marginal ear veins 10 days after the last antibiotic injection.

Agglutinin Titers.—Levels of agglutinating antibody were determined using 3 by 0.5 in. tubes containing serial doubling dilutions of serum in 0.5 ml. amounts. Aliquot volumes of standardized suspensions of the appropriate organisms were added, and the tubes were incubated at 52° C. overnight and then allowed to stand at room temperature for 4 hours before reading.

Agglutinin Adsorption Tests.—A series of agglutinin adsorption tests were carried out on (1) antisera containing *S. typhosa* somatic "O" antibody and (2) antisera containing Vi antibody, from both untreated rabbits and rabbits treated with chloramphenicol. Suspensions for adsorption were grown on nutrient agar on standard 90 mm. plates for 18 hours. The growth on each plate was washed off in 1.0 ml. of sterile 0.85 per cent saline. The organisms were then washed 3 times in 8.0 ml. of saline and finally resuspended in 1.0 ml. of fresh saline. One milliliter quantities of the sera, diluted to 1:20 of the initial agglutinin titer, were added. The mixtures were incubated at 37° C. for 4 hours and the organisms were then centrifuged at high speed. Adsorption of sera was repeated on two occasions. Serial doubling dilutions of the final supernatant sera were then tested for agglutinin titers using standard suspensions in the usual way. In addition to adsorbing the two types of sera with suspensions of normal *Esch. coli* and *S. typhosa* organisms, it was decided to determine whether or not treatment of the organisms in vitro with chloramphenicol would alter their ability to adsorb out the appropriate antibodies. Organisms treated with chloramphenicol were prepared by harvesting the growth from overnight nutrient agar plate cultures, in sterile normal saline. Suspensions were then divided into 2 aliquot portions and chloramphenicol in final concentrations of 50 and 100 μg per milliliter, respectively, was added to each tube. The tubes were then incubated at 37° C. for 3 hours. The organisms were then well washed in several changes of saline to eliminate residual chloramphenicol and were resuspended in fresh saline to a final concentration of 10^8 organisms per milliliter. Adsorption of sera was then carried out as described.

Bactericidal Effect of Sera.—In vitro tests of the bactericidal power of the sera were carried out using antisera against all the strains employed. Each rabbit was bled the day before the tests were carried out and the serum was separated after overnight storage at 4° C. The sera were then diluted 1:10 in sterile 0.85 per cent saline and then inactivated at 56° C. for 30 minutes. This procedure removes nonspecific bactericidal effects[2] and also destroys serum properdin.[3]

Complement was obtained by pooling the sera of healthy guinea pigs following cardiac puncture. Preliminary adsorption of the sera was carried out by dividing the pooled serum into 3 batches and adsorbing each with one of the 3 bacterial strains. The adsorbing strains of *S. typhosa* 0.901 and *S. typhimurium* were killed by heat at 100° C. for 30 minutes and the Vi-containing *Esch. coli* strain by 50 per cent ethanol at 37° C. for 20 hours.[4]

Tests were carried out by placing 0.5 ml. of serum in sterile 3 by 0.5 in. tubes along with 0.1 ml. of inoculum. The inoculum consisted of a $1:10^6$ dilution on an 18-hour broth culture. Tubes were incubated at 37° C. for 1 hour to sensitize the cells. Pooled complement was then added in 0.1 ml. amounts. Complement control tubes containing complement, inoculum, and saline, and inoculum control tubes containing saline and inoculum were included. Tubes were incubated at 37° C., and viable organism counts were carried out at intervals by the technique of Miles and Misra.[5]

Mouse Protection Tests.—The relative protective power of the two types of antisera against *S. typhimurium* was established by mouse protection tests. Fifty-six mice were

used for each test. The mice averaged 25 grams in weight, and were free from natural Salmonella infection. Amounts of 0.5 ml. of pooled sera from 3 immunized and chloramphenicol-treated rabbits were injected intraperitoneally into 28 mice. Aliquot volumes of pooled sera from immunized but nonantibiotic-treated rabbits were injected into the remainder. Serial tenfold dilutions of an 18-hour agar slope culture of *S. typhimurium* were prepared in sterile normal saline, ranging from 1:10 to $1:10^6$. Half-milliliter quantities of each were then added to 4.5 ml. of a sterile 5 per cent solution of granular mucin, giving final bacterial dilutions of $1:10^2$ to $1:10^7$. The mice were divided at random into groups of 4 from each serum treatment, and 0.5 ml. amounts of the varying bacterial dilutions were injected intraperitoneally into each member of each group one hour after injection of the sera. The groups of 4 were kept in separate cages and deaths were recorded during the following 30 days. Cumulative survivor and death totals were then calculated and the LD_{50} dose was determined by the method of Reed and Muench.[6]

RESULTS

A. Agglutinin Titers of Antibiotic-Treated and Immunized Control Animals.—In Table I are detailed the quantitative results of agglutinin content of a series of sera from rabbits immunized with the 3 bacterial strains. These results are similar to previous observations which we have made concerning the production of antibody to both bacterial and nonbacterial antigens in the presence of antibiotic, and show that under the experimental conditions employed there appeared to be no difference in the final quantitative titers of the two groups.[7]

TABLE I. QUANTITATIVE AGGLUTININ TITERS OF IMMUNIZED RABBITS

ORGANISM	ANTIBODY TITERS*	
	(A) ANTIBIOTIC GROUP	(B) NONANTIBIOTIC GROUP
S. typhosa	rabbit	rabbit
	1 - 640	4 - 2,560
	2 - 1,280	5 - 1,280
	3 - 1,280	6 - 1,280
S. typhimurium	rabbit	rabbit
	1 - 1,280	4 - 1,280
	2 - 1,280	5 - 1,280
	3 - 640	6 - 1,280
Esch. coli	rabbit	rabbit
	1 - 5,120	4 - 5,120
	2 - 5,120	5 - 5,120
	3 -10,240	6 - 5,120

Figures are the reciprocals of the final serum dilutions.
*(A) = treated with chloramphenicol; (B) = no antibiotic treatment.

However, the literature contains a large number of conflicting reports regarding the effect of antibiotics on quantitative antibody production, results ranging from marked inhibition to no effect, and even increased antibody production. Such results are perhaps not altogether unexpected since variations in the type of antibiotic (i.e., whether primarily bacteriostatic or bactericidal in action), the nature of the host, and the type of parasite can all influence the final results.

Our experience with clinical cases of enteric fever suggests that there is a delay in the production of antibody, where treatment is begun in the first few

days of illness, but that the final titers are not significantly lower than in nontreated cases. Where treatment is delayed till about the sixth to eighth days of illness then the antibiotic appears to have no influence on subsequent antibody formation.

TABLE II. ADSORPTION OF TYPE O ANTIBODY

ANTISERUM	ADSORBED BY	AGGLUTININ TITERS AFTER ADSORPTION	
		0.901	0.901*
(1) *S. typhosa* O	0.901	Neg.	Neg.
	0.901*	Neg.	Neg.
(2) *S. typhosa* O†	0.901	Neg.	Neg.
	0.901*	Neg.	Neg.

*Organisms treated with chloramphenicol.
†Sera from chloramphenicol-treated immunized rabbits.

TABLE III. ADSORPTION OF VI ANTIBODY

ANTISERUM	ADSORBED BY	AGGLUTININ TITERS AFTER ADSORPTION	
		VI-N*	VI-C†
(a) Vi	Vi*	Neg.	Neg.
	Vi†	64	64
(b) Vi‡	Vi*	Neg.	Neg.
	Vi†	64	64

Figures are reciprocals of final serum dilutions.
*Normal Vi organism suspension.
†Vi suspension treated with chloramphenicol.
‡Sera from chloramphenicol-treated immunized rabbits.

B. Agglutinin Adsorption Tests on Sera From Chloramphenicol-Treated and Untreated Immunized Rabbits.—In Tables II and III are shown the results of adsorption of agglutinins in the case of antisera containing *S. typhosa* somatic type O antibody and Vi antibody, respectively. In the case of somatic type O antibody it was possible to show complete removal of agglutinins from both groups of sera by adsorption with either normal organism suspensions or organisms treated with chloramphenicol. Treatment with chloramphenicol at the concentrations and time stated did not appear to affect the somatic antigen sufficiently to alter its ability to adsorb antibody. Similarly, it was found that normal Vi-containing organisms would completely remove Vi antibody from either the chloramphenicol-treated group sera or the nontreated group sera. This suggests that the type of antibody formed in both groups has similar combining powers for the respective adsorbing strains. Assuming that chloramphenicol may cause some alteration in the determinative pattern of the antigen the resulting antibody produced might reflect such alteration in the spatial orientation of its molecular structure. Thus, the ability of such antibody to combine with the normal organisms in vitro might be reduced. Conversely, however, any alteration produced in the antigenic structure of the organism might merely be of a quantitative nature in which case qualitative alterations in the type of antibody formed would not be expected.

In the case of the Vi-containing adsorbing suspensions, treatment with chloramphenicol resulted in a failure of the organisms to remove completely the Vi antibody from both groups of sera. The reason for this failure is not

clear but is possibly due to a reduction in the amount of Vi antigen. Burgio and Rosano,[8] for example, found that strains of *S. typhosa* grown in media containing chloramphenicol exhibited a reduction in agglutinability with anti-O and anti-H sera but an enhanced agglutinability with anti-Vi sera. As Felix and Pitt[9] have shown that the greater the amount of Vi antigen in *S. typhosa* strains the lower the titer in the Vi agglutination reaction.

C. Bactericidal Effect of Immune Sera From Chloramphenicol-Treated and Untreated Animals.—The results of in vitro bactericidal tests conducted on the two groups of sera are shown below (Table IV).

TABLE IV. BACTERICIDAL SERUM TESTS IN VITRO

ORGANISM	TIME OF SAMPLING (HOURS)	SERUM*						INITIAL INOCULUM AT ZERO TIME
		A	B	C	D	E	F	
S. typhi-murium	1	5	3	4	8	10	6	30
	2	1	2	2.5	1	3	2	
	3	1	Neg.	Neg.	0.5	Neg.	1	
	6	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	
	24							
S. typhosa	1	20	11	3	5	22	18	120
	2	1	1	Neg.	1	2	2	
	3	Neg.	Neg.	Neg.	Neg.	1	1	
	6	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	
	24							
Esch. coli	1	50	56	60	55	62	60	160
	2	30	40	44	40	42	45	
	3	2	Neg.	Neg.	Neg.	1	Neg.	
	6	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	
	24							

Figures represent numbers of colonies per 0.02 ml. volume after overnight incubation.

Saline and complement inoculum control tubes showed no reduction in numbers of organisms during the period of test.

*Sera A, B, and C from chloramphenicol-treated rabbits. Sera D, E, and F from non-antibiotic-treated rabbits.

The in vitro bactericidal power of the two groups of sera appeared to be practically identical and the findings do not suggest that chloramphenicol in any way results in the production of antisera with less protective functions than normal type immune sera. However, one must bear in mind the possible limitations of in vitro bactericidal tests since the additional in vivo defense factors which exist are not possible to simulate in the in vitro experimental model.

D. Mouse Protection Tests Using S. typhimurium Immune Sera From Chloramphenicol-Treated and Untreated Animals.—Calculation of the LD_{50} dose of *S. typhimurium* for the two serum-treated groups of mice gave an interpolated value between 10^3 and 10^4 for both groups. The protective value of the antibody in the chloramphenicol-treated group of rabbits did not appear to be less than that in the nontreated group. The mouse protection tests were repeated, using a different strain of *S. typhimurium*, with identical results.

DISCUSSION

The effect of antibiotic therapy on the humoral response in infectious disease states depends to a large extent on the time at which therapy is begun. If

given during the first few days of illness there often appears to be a delay in antibody production. Such findings have been reported by Smadel[10] in the case of chloramphenicol therapy in scrub typhus. Our own observations in the treatment of typhoid fever with chloramphenicol would support this finding.[1] However, since complete eradication of the antigen is not to be expected, particularly where the antibiotic has primarily a bacteriostatic action, the final attainable titers are usually not lower than those to be found in nontreated cases. Where therapy is delayed till after the first week of illness, in the case of enteric infections, the effect of the antibiotic on the subsequent production of antibody is probably small. In the relapse phase of typhoid fever we have been unable to establish any correlation between the occurrence of relapse and the quantitative amount of antibody present.[1] Clinical relapse may occur in spite of high antibody levels, and the high relapse rates which have been reported in patients inadequately treated with chloramphenicol cannot be satisfactorily explained on the basis of lack of antigenic stimulus with consequent lack of antibody formation.

The relative importance of humoral versus cellular and other mechanisms of defense in many infectious disease states is still a matter of confusion. In a few instances, for example, the effect of antibody against the specific soluble substance of pneumococci and the antibody response in certain viral infections, it appears that humoral factors are of primary importance. In the case of the enteric group infections the situation is less clear. The ease with which agglutinating antibody can be demonstrated has tended to focus attention on the importance of humoral aspects of protection. Clinical observations, however, are not entirely in agreement with this concept. It is well known, for example, that certain patients never develop demonstrable antibodies against either the somatic or flagellar antigens. Others again show only a type H antibody response—a type of antibody which has no protective function at all. We have shown that roughly one-third of bacteriologically proved cases of typhoid fever never exhibit titers as high as the minimum levels we have suggested as being applicable to this part of the world.[11] Such patients, however, appear to be able to overcome the infection as well as those who exhibit high antibody levels. It is true that certain patients may develop an incomplete type of antibody which will only be detected by the use of an anti-human globulin technique and that such antibody may well have a protective function. However, the fact that clinical relapse can occur with high levels of agglutinating antibody suggests that humoral factors are a relatively poor defense mechanism in enteric fever, and thus one must look elsewhere for an adequate explanation of the effect of chloramphenicol in increasing the relapse rate when given for too short a period of time.

The present results seem to indicate that there is little, if any, difference in the qualitative nature of the antibody produced against strains of the Enterobacteriaceae family in groups of animals treated with chloramphenicol and in those animals not treated. It may be that the methods of detection of such qualitative differences are too crude to determine minor changes in the spatial

orientation of the antibody. This may apply especially in the case of in vitro bactericidal tests since the experimental model employed in such tests may not allow for additional factors operating in vivo. We have recently attempted to study the qualitative nature of such antibody by means of agar gel double diffusion techniques, using antigenic extracts prepared according to the method of Webster and associates.[12] Here again preliminary results show that the precipitation patterns observed with both groups of sera are identical in all respects.

A number of workers have observed changes in antigenic structure of organisms as the result of antibiotic therapy. Such investigations have usually been carried out by observing alterations in antigenic make-up following exposure of the organisms to varying concentrations of antibiotic for varying periods of time, or by studying the antigenic structure of descendents of organisms grown in media containing subinhibitory concentrations of antibiotic. Servant,[13] for example, has found that treatment of certain strains of the Enterobacteriaceae family with dihydrostreptomycin may reduce the amount of extractable polysaccharide by as much as 300 per cent, as compared with normal organisms. We have cited the work of Burgio and Rosano[8] who found that strains of *S. typhosa* grown in media containing chloramphenicol exhibited reduced agglutinability with anti-O and anti-H sera and enhanced agglutinability with anti-Vi sera. Farhi and Lamensans,[14] on the other hand, found that *S. typhosa* strains in contact with chloramphenicol for a period of 5 hours became hyperagglutinable with anti-O sera and hypoagglutinable with anti-Vi sera. These latter workers also reported that the antibodies produced in response to the modified organisms were less protective than antibodies produced against the normal organisms. Braun and associates[15] found that strains of brucella grown in increasing concentrations of penicillin produced descendents differing in antigenic structure from the original strains. Antigenic variations of this type may conceivably be either qualitative and/or quantitative in nature. In the former case, qualitative changes in the antibody produced will depend on the experimental conditions employed with respect to the concentrations of antibiotic used and the time of interaction with the organisms. Consequently it may not be possible to apply the results of in vitro experiments to conditions pertaining in vivo since in the latter the organisms may be protected from the effect of the antibiotic to a large extent, for example, by their intracellular position. Quantitative changes in bacterial antigenic components, on the other hand, may or may not result in a change in the total amount of antibody produced.

It seems unlikely that the high relapse rates in enteric fever, associated with inadequate chloramphenicol therapy, are related to either a quantitative failure of antibody production or a qualitative change in the nature of the antibody. It would seem that the host cellular factors are of importance, and we have previously discussed the possibility that chloramphenicol may increase the number of intracellular forms of the organism.[1] In this connection Choremis and associates[16] found a 14 per cent relapse rate in 100 cases of enteric fever

treated with chloramphenicol as compared with only 7 per cent in a further 100 cases treated with similar doses of chloramphenicol but who were given cortisone in a dose of 1 to 2 mg. per kilogram daily for up to 10 days. Cortisone has a depressing effect on phagocytosis and consequently may reduce the number of intracellular organisms available for initiation of the relapse phase.

SUMMARY

An investigation of certain aspects of the qualitative nature of antibody produced in animals treated with chloramphenicol failed to reveal any differences as compared with antibody produced in nonantibiotic-treated animals. Immunizing strains employed were *Salmonella typhosa, S. typhimurium,* and a strain of *Escherichia coli* rich in Vi antigen. It is suggested that the high relapse rates[1] found in inadequately treated cases of enteric fever are not related to a failure of antibody formation or to qualitative alterations in the type of antibody.

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ANTIBODY RESPONSE TO INTRACUTANEOUS AND SUBCUTANEOUS INFLUENZA VACCINATION

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RECOMMENDATIONS for the route of vaccine administration immediately prior to the Asian influenza epidemic of 1957 had to be made on contradictory evidence. Van Gelder and associates[1] and Bruyn and co-workers,[2] using older influenza vaccines, of unspecified chicken cell agglutinating (CCA) units, had found the intracutaneous inoculation to produce a superior antibody response relative to the subcutaneous inoculation in tests employing both routes in adults. Later, in 1956, Woolridge and Seal,[3] in similar tests with monovalent 500 CCA unit vaccines found the subcutaneous route to be superior. Since the 1957 epidemic, Hilleman and associates,[4] using vaccines of 160, 185, and 500 CCA units, reported that no major difference was evident between the two routes of vaccine administration. The wide differences in the initial titer levels among these groups, however, indicate a lack of comparability and make their results difficult to interpret. In addition, other investigators had reached similar conflicting conclusions from studies using only one or the other route. Our present study essentially confirms previous work, and also the resulting divergent views, and may indicate the reason for confusion in this area.

METHODS

In February, 1958, we inoculated 168 Navy recruit volunteers from the United States Naval Training Center, Great Lakes, Illinois. The men were randomly assigned to groups which received an inoculation by one or the other route of undiluted, 1:10, 1:100, and 1:10,000 saline dilutions of Asian influenza virus vaccine (A/JAP/305-57) containing about 800 CCA units per milliliter. Men receiving the vaccine by the subcutaneous route were given 1 ml. injections in the upper arm using a ¾ inch 23 gauge needle. These inoculations were possibly intramuscular in thin men. The men receiving intracutaneous inoculations were given 0.1 ml. injections in the forearm. Presumably, some leakage occurred into subcutaneous levels. A 1 ml. formalinized saline inoculation was given subcutaneously to a placebo group. Thus, we studied 9 groups which are designated in terms of the CCA unit each received, i.e., subcutaneous: 800, 80, 8, 0.08, and 0.000 (placebo); and intracutaneous: 80, 8, 0.8, and 0.008.

The men were bled prior to inoculation and again 21 days later. The initial and 21-day sera were titrated "blind" in a single hemagglutination inhibition test and again using the complement-fixation test. The test proposed by the Committee on Standard

From United States Naval Medical Research Unit No. 4, Research Project NM 52 05 04.5.
The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.
Received for publication Sept. 19, 1958.

Serological Procedures[5] was used to determine the hemagglutination inhibition antibody response to the vaccine. The sera and virus antigen were treated with KIO[4] according to the method described by the United States Public Health Service.[6] The modified Kolmer technique was employed for the complement fixation test.[7]

RESULTS

The random selection, stratified to give 5 groups with double the number of men of the other 4 groups resulted in groups with reasonably comparable initial antibody titer levels to Asian influenza virus. Since the HI and CF antibody titers tended to be proportional in all aspects, only the former are discussed in this report. Table I gives the initial HI antibody titers, geometric mean titers and the number of men in each group. An analysis of variance of these data indicates that the twofold variation in the initial titers of the groups is well within errors of sampling. The high antibody titer levels, after the 1957 influenza epidemic, indicate that the conclusions of this study apply to an anamnestic response rather than to a primary response.

TABLE I. NUMBER OF NAVY RECRUITS AT VARIOUS INITIAL HI ANTIBODY TITER LEVELS BY VACCINE GROUP, GREAT LAKES, ILL., FEBRUARY, 1958

RECIPROCAL OF TITER	1 ML. SUBCUTANEOUS GROUPS (CCA UNITS)				0.1 ML. INTRACUTANEOUS GROUPS (CCA UNITS)				PLACEBO
	800	80	8	0.08	80	8	0.8	0.008	0.000
<8	5	5	3	0	3	0	1	2	1
8	3	1	3	0	6	5	3	0	2
16	7	5	1	1	6	4	2	1	0
32	3	6	5	4	4	13	2	4	3
64	4	5	8	3	6	3	3	3	2
128	2	3	2	0	2	1	0	0	4
256	0	2	1	0	0	0	0	0	0
No. recruits	24	27	23	8	27	26	11	10	12
Geometric mean titers	18	28	32	38	21	25	19	24	38

Data for average tubes, or logarithms of antibody titer change, are shown in Fig. 1. An analysis of variance of these data indicates a substantial variation among the groups ($P<0.01$). The slight average increase in the placebo group (indicated as zero CCA unit in Fig. 1) may reflect some concurrent influenza infection; presumably natural infections occurring in the other groups, if at all present, would not be greater than for the placebo.

It is evident from Fig. 1, that the intracutaneous route of vaccine administration was accompanied by a superior antibody response with dosages of about 50 CCA units per milliliter or less. With vaccine of more than about 50 CCA units per milliliter, and inoculations of 1 ml. subcutaneously and 0.1 ml. intracutaneously, the subcutaneous route was superior, at least in Navy recruits. Hence, by interpolation, we might expect a subcutaneous inoculation of about 50 CCA units of vaccine to produce an antibody response identical to that of an intracutaneous inoculation of about 5 CCA units. Older vaccines very likely contained fewer CCA units than present vaccines. Further, where these vaccines were mixtures of several viruses, the CCA unitage of a particular virus

would be a small fraction of the total CCA unitage of the vaccine. Hence, the reported superiority of the intracutaneous route[1, 2] may very well have been due to the small amount of CCA units of specific antigen in the vaccine used. Monovalent vaccine inoculations of more than about 50 CCA units,[3] tend to result in relatively greater antibody production when given by the subcutaneous route. Our studies suggest that the relation between dosage in antibody response is linear in the case of the intracutaneous route and curvilinear (or sigmoid) in the case of the subcutaneous route. There may be a quantitative variation in this relation between dosages and route from viral strain to strain, and perhaps from one population of test subjects to another. However, it seems reasonable to interpret the previously conflicting reports as being due to the variation in CCA unitage and to the difference in antibody response, as noted in this report, which seems to vary with dosage and inoculation route.

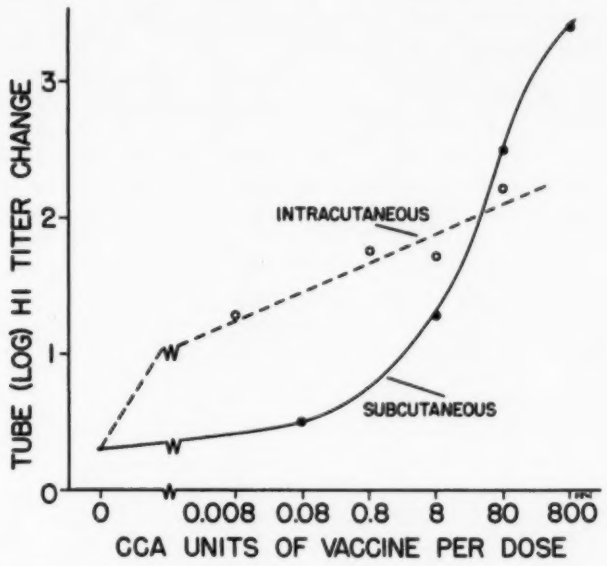

Fig. 1.—Average tube (or $\log_2$) HI antibody titer change to Asian influenza 21 days after inoculating Navy recruits by various routes and dosages of Asian influenza virus vaccine, Great Lakes, Ill., February-March, 1958.

SUMMARY

The antigenic response of male United States Navy recruits to influenza vaccine was studied. The intracutaneous and subcutaneous routes of inoculation were employed using undiluted vaccine and various dilutions to 1:10,000. The antigenic response following intracutaneous inoculation tended to be proportional to the logarithm of the dose (a straight line); while the responses to the subcutaneous route tended to show a sigmoid relation, crossing the line for intracutaneous route at about 50 CCA units. It is suggested that the previous

differences noted by other investigators, favoring one or the other routes of vaccine administration, are in part due to variation in the vaccine potency (particularly to a specific antigen in the case of polyvalent vaccines) and the biological difference, herein noted, between these two routes of vaccine administration.

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UNIVERSAL SEROLOGIC REACTION IN PLASMACYTIC MYELOMA

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LAWSON and co-workers[1] reported that patients with multiple myeloma show either a complete absence or a marked deficiency in natural antibodies. The antibodies studied included agglutinins for typhoid and other enteric organisms and for sheep erythrocytes and, also, type-specific agglutinins for human A and B erythrocytes. They state that "the immunologic abnormality demonstrated in multiple myeloma is considered to be the result of abnormal function of the malignant plasma cells whereby deranged protein synthesis results in the production of abnormal proteins at the expense of normal proteins, including antibody protein."

Wiener and associates[2] stated recently: "Many studies have been attempted to determine the nature of the serum globulin responsible for the classical findings of multiple myeloma, and to indicate whether it represents an excess of a normal serum constituent or is in fact an abnormal protein specific for the disease. The consensus, based on experimental studies, suggests that an abnormal protein is produced by the pathologic plasmacytes of bone marrow." Putnam[3] also reported that in multiple myeloma "the abnormal globulins accumulate as if they were made by a defective assembly-line that had lost the ability to produce the diverse spectrum of normal globulins."

The absence of natural antibodies in plasmacytic myeloma, combined with the existence of abnormal globulins in this disease, raised the question whether what appears to be an antibody to lipids, detected in the universal serologic reaction, would also be absent. This serum reaction, based on flocculation, has been found to be positive in all human beings and animals tested thus far. As in the case of other antibody reactions, only the globulins of serum give positive flocculation results, the albumins giving completely negative results.[4, 5]

The universal reaction is unique in several respects. Although universally positive, the reaction is readily differentiated on the basis of differences in serologic patterns. These differences are based on the intensity of the flocculation reactions which may be weak, moderate, or strong, and also on the arrangement of the flocculation pattern. In health, these patterns tend to be constant. For example, a person showing a given pattern of strong flocculation gives the same pattern on repeat examinations at different intervals.

In the chronic diseases studied thus far, including syphilis, tuberculosis, malaria, and leprosy, the universal reaction increases in intensity over the

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This study was aided in part by the Difco Laboratories, Inc., Detroit, Mich., and by the Wenner-Gren Foundation for Anthropological Research, New York, N. Y.

Received for publication Sept. 12, 1958.

normal flocculation level. Exceptions are forms of tuberculosis or leprosy in which the disease is widely disseminated, when the reaction is generally weak—due presumably to inability of the patients to produce antibodies.[4]

Kahn believes that the universal reaction in health is the result of an autoantigen-antibody reaction to lipids. His theory is that lipids liberated in the course of normal metabolism undergo such conjugation with protein as to become autoantigenic, calling forth autoantibody formation to lipids. Under conditions of tissue breakdown, especially in chronic diseases, the increase in lipid liberation leads to a corresponding increase in autoantibody formation and to universal reactions of increased intensity.

TABLE I. UNIVERSAL SEROLOGIC REACTION WITH NORMAL HUMAN SERUM (SEVEN QUANTITATIVE SETUPS OF SERUM DILUTIONS WITH LIPID ANTIGEN)

SERIAL DILUTIONS OF SERUM	PER CENT NaCl SOLUTIONS EMPLOYED IN SERUM DILUTIONS						
	0	0.15	0.6	0.9*	1.2	1.8	2.1
Readings made without incubation of serum-antigen suspension mixture							
1:256	–	–	–	–	–	–	–
1:64	–	–	–	–	–	–	–
1:16	–	–	–	–	–	–	–
1:8	–	–	–	–	–	–	–
1:4	–	–	–	–	–	–	–
1:2	–	–	–	–	–	–	–
Und.	–	–	–	–	–	–	–
Readings after 4 hours' incubation in an icebox							
1:256	–	–	–	–	–	–	–
1:64	–	–	–	–	–	–	–
1:16	–	–	–	–	–	–	–
1:8	–	–	–	–	–	–	4
1:4	2	4	–	–	–	4	4
1:2	4	4	–	–	–	4	4
Und.	4	4	–	–	–	4	4
Readings after 24 hours' incubation in an icebox							
1:256	–	–	–	–	–	–	–
1:64	4	–	–	–	–	–	4
1:16	4	4	–	–	–	4	4
1:8	4	4	–	–	–	4	4
1:4	4	4	–	–	–	4	4
1:2	4	4	–	–	–	4	4
Und.	4	4	–	–	–	4	4

*The quantitative setup with 0.9 per cent NaCl solution, read without incubation, is similar to a quantitative Kahn reaction.

METHODS

Before presenting the findings of the universal serologic reactions in plasmacytic myeloma, the serologic technique will first be briefly presented, and the results given by normal individuals illustrated.

The technique of the universal reaction consists of 7 quantitative setups of serum dilutions plus Kahn antigen suspension. The first setup of the serum dilutions is prepared serially with distilled water; the second with 0.15 per cent NaCl solution; the third with 0.6 per cent NaCl solution; the fourth with 0.9 per cent; the fifth with 1.2 per cent; the sixth with 1.8 per cent; and the seventh with 2.1 per cent NaCl solution. These serum dilutions in 0.15 ml. amounts are mixed with antigen suspension in 0.025 ml. amounts (ratio 6:1). The mixtures are shaken for 3 minutes and the flocculation results read immediately. A second reading is made after 4 hours' incubation at 4° C., and a third reading after 24 hours at the same temperature.

Table I illustrates a weak universal serologic reaction given by a normal individual. The flocculation results of this reaction are presented graphically by the cross-hatched areas in Graph 1 of Fig. 1. The figure also illustrates a moderately strong reaction (Graph 2) in which flocculation is marked after 24 hours at cold incubation, and a strong reaction (Graph 3) in which flocculation is also marked after 4 hours at cold incubation.

The experimental plan was to draw blood specimens from patients with plasmacytic myeloma and examine them with the universal serologic technique. Because the universal reaction was determined in this laboratory at the same time in normal individuals and in patients with diseases other than plasmacytic myeloma in connection with other serologic studies, there were always an ample number of controls employed.

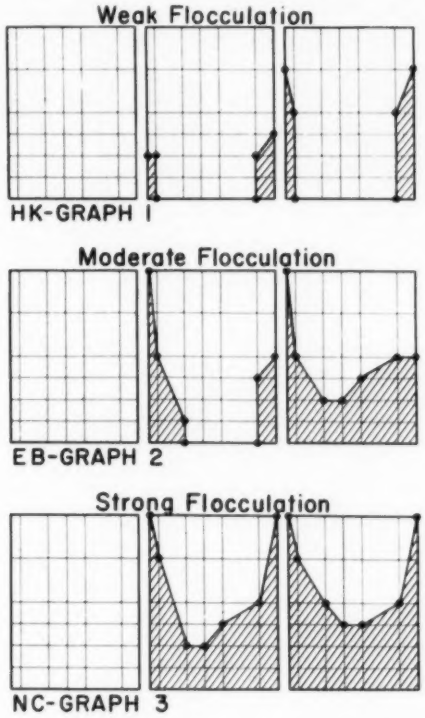

Fig. 1.—Universal serologic reactions in normal human beings.

RESULTS

Table II lists 12 patients with a diagnosis of plasmacytic myeloma in which the universal reaction gave negative results. The main data which led to the diagnosis, including initial symptoms, total serum protein, serum globulin (Howe method), paper electrophoresis, hemoglobin, Bence Jones protein, marrow aspiration, and x-ray picture, are summarized in the table. The table also lists 4 patients with plasmacytic myeloma who gave very weakly positive universal reactions. It can be seen from Fig. 2 that the flocculation in these 4 patients is

TABLE II. CLINICAL DATA OF 12 PATIENTS WITH PLASMACYTIC MYELOMA WHO GAVE NEGATIVE UNIVERSAL REACTIONS AND OF 4 PATIENTS WHO GAVE WEAK REACTIONS

PATIENT	AGE	SEX	INITIAL SYMPTOM	TOTAL SERUM PROTEIN (GM. %)	GLOBULIN* (GM. %)	PAPER-ELECTROPHORESIS	HGB (GM. %)	BENCE JONES PROTEIN	MARROW ASPIRATION	X-RAY	UNIVERSAL REACTION
G. C. M.	40	M	Low back pain	11.1	8.1	49 per cent gamma globulin	12.6	Not tested	Extensive replacement with neoplastic plasmacytes	Demineralization of spine with irregular lytic lesions of spine, pelvis, and femurs	Neg.
J. Q.	58	M	Bulbous skin eruption	9.2	5.6	53 per cent beta globulin	9.2	Neg.	20 per cent plasmacytes, many atypical	No skeletal lesions demonstrated	Neg.
G. P.	46	F	Pain in left shoulder	8.0	4.5	38 per cent beta globulin	12.3	Neg.	20 per cent plasmacytes, mostly atypical	Fracture (''pathologic'') surgical neck, left humerus	Neg.
W. B.	39	M	Back pain	12.6	9.9	79 per cent beta globulin	10.6	Pos.	Aspirations of sternum and ilium showed no myeloma. Iliac biopsy showed plasmacytoma	Multiple lytic lesions of skull, ribs, vertebrae, femur, pelvis	Neg.
H. P.	60	F	Back pain	8.0	4.2	''M'' peak 34.3 per cent	12.0	Neg.	20 per cent myeloma cells	Lytic lesions of skull and left femur	Neg.
O. E.	53	M	Back pain	11.4	7.9	Gamma globulin increased	11.5	Neg.	Extensive replacement with atypical plasmacytes	Demineralization of spine, collapse of D_{10}	Neg.
I. M.	55	F	Back pain	6.4	2.0	Slight increase of alpha-2 globulin	12.6	Neg.	Extensive replacement with atypical plasmacytes	Generalized demineralization of spine. Compression of D_9 and D_{11}	Neg.
B. J.	44	M	Pain in ribs	7.7	1.7	Slight increase of alpha-2 and gamma globulin	13.4	Neg.	Almost complete replacement with abnormal plasmacytes	Multiple lytic lesions of pelvis, skull, femurs, and left humerus. Spiral fracture of left humerus	Neg.

S. S.	69	M	Anterior chest pain	6.0	1.4	Increased beta globulin	9.6	Pos.	Plasmacytes increased in number. Most were well differentiated but some atypical and poorly differentiated	Generalized demineralization of spine. Anterior wedging of D_6. Lytic lesions of pelvis and femurs	Neg.
L. O.	61	M	Back pain	13.3	10.6	Increased beta globulin	7.4	Neg.	Myeloma cells of immature type	Generalized demineralization of skeleton. Lytic lesions of skull, ribs, clavicles, pelvis, and femurs	Neg.
H. W.	63	F	Back pain	10.9	6.3	"M" peak between beta and gamma globulin	6.8	Neg.	Extensive replacement with atypical plasmacytes	Generalized demineralization of skeleton. Collapse of D_{11}, D_{12}, and L_1	Neg.
T. M.	72	M	Ease of fatigue (anemia)	6.2	1.3	Slight increase of beta globulin	9.3	Neg.	80 per cent well-differentiated plasmacytes	Demineralization of lumbar spine and decreased vertical height of D_{12}	Neg.
P. M.	62	M	Chest pain	7.8	2.7	Questionable elevation of alpha-2 globulin	11.1	Pos.	Hypocellular specimen, but atypical plasmacytes with hyperchromatic nuclei present	Lytic lesions of skull, ribs, clavicles	Weakly pos.
H. A.	55	M	Asymptomatic in regard to myeloma. Diabetes mellitus	13.6	10.8	Gamma globulin increased	8.7	Neg.	Plasmacytes increased in number and atypical in appearance	No skeletal lesions demonstrated	Weakly pos.
A. C.	43	M	Low back pain	7.0	2.9	31 per cent gamma globulin	5.8	Not tested	Mostly atypical plasmacytes	No skeletal lesions demonstrated	Weakly pos.
T. W.	68	M	Chest and back pain	12.0	8.9	Beta globulin increased	12.1	Neg.	Almost complete replacement of normal cells by abnormal plasmacytes	Lytic lesion of tenth thoracic vertebra	Weakly pos.

*Howe method.

unusually weak. The circumstances under which these patients were studied serologically were such as to permit only one examination and at a time in which they were undergoing treatment. Had we had the opportunity to make several serologic examinations during the disease, some of the reactions might have been completely negative. It is possible that, in some patients with plasmacytic myeloma, treatment exerts a special effect on the universal reaction causing them to regain some degree of reactivity.

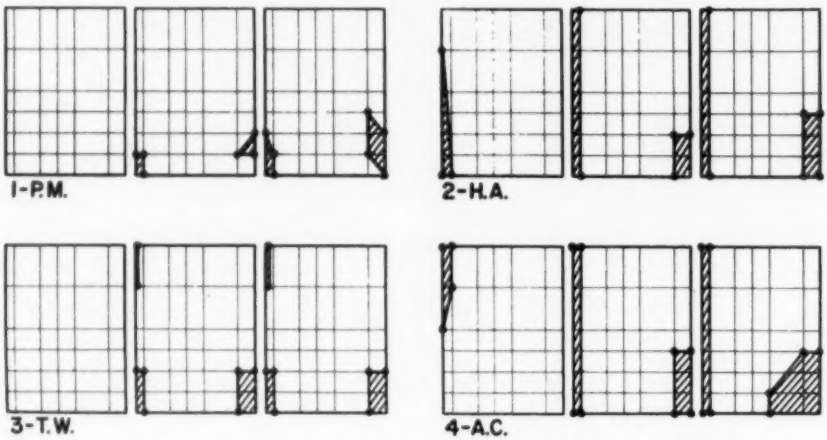

Fig. 2.—Weak universal reactions in 4 patients with plasmacytic myeloma.

Six additional patients are discussed in whom the diagnosis of plasmacytic myeloma was questionable or ruled out on the basis of clinical and laboratory findings. Some of these gave quite strong universal reactions. These patients are the type which confront the physician with the problem of deciding whether to treat for myelomatosis or for some other condition. The case reports of these patients are briefly summarized below, and Fig. 3 presents their universal reactions.

CASE 1 (A. W.).—This 68-year-old housewife was admitted to the University of Michigan Hospital because of recurrent pain in her right hip during the year previous to admission. The pain was dull, aggravated by sitting, and there was slight radiation of the pain into the right leg. The pain increased in frequency until it was present almost continuously during the 4 months before admission. There had been a 15-pound weight loss during the 6 months before admission.

Examination revealed a slightly obese woman who did not appear ill. The blood pressure was 160/80 mm. Hg; the pulse was 72 per minute. The general physical examination was not otherwise unusual.

Urinalysis was within normal limits. There was no Bence Jones protein in the urine. The peripheral blood showed no distinctive abnormality. The hemoglobin was 12.3 Gm. per 100 ml. and the hematocrit 38 per cent. No diagnosis was warranted from sternal marrow examination. Total serum protein was 6.6 Gm. per 100 ml. and the distribution of the protein fractions was normal on paper electrophoresis.

X-ray examination of the spine showed a vertical radiolucency of the ninth dorsal vertebra which suggested the presence of a hemangioma. There was a lytic defect in the right side of the sacrum. A cordotomy was done for relief of pain.

Biopsy of the sacral region revealed a fibrosarcoma. The universal reaction was weakly positive.

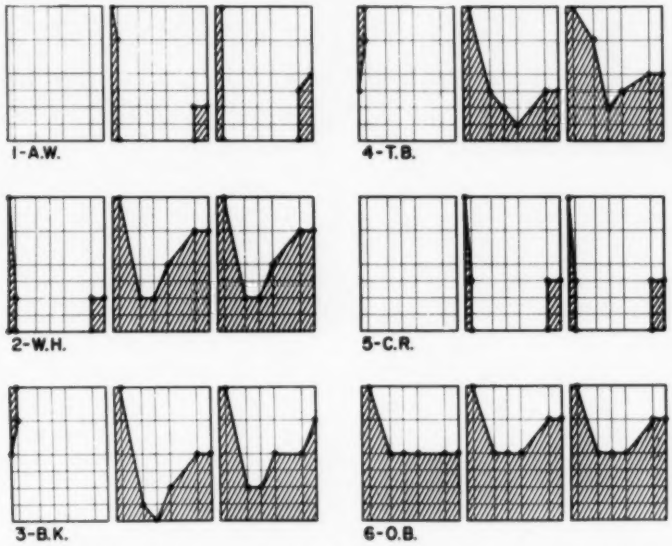

Fig. 3.—Universal reactions in 6 patients with doubtful diagnosis of plasmacytic myeloma.

CASE 2 (W. H.).—This 56-year-old housewife was referred to University Hospital because of back pain of 1 week's duration. She sustained back trauma 15 years before admission and had recurrent pain localized in the low back. This had not been severe enough to require treatment previously. One week before admission, she noted onset of severe pain in her back while getting into bed. Pain was persistent to the time of admission. It was localized in the back, aggravated by walking but not aggravated by coughing. The patient had been known to have hypertension and diabetes for 10 years. She had had no menstrual periods for 2 years.

Physical examination showed a moderately obese woman in no acute distress. The blood pressure was 220/120 mm. Hg. The pulse was 80 per minute. The heart was slightly enlarged to percussion. There was a soft systolic murmur at the cardiac apex.

Urinalysis was within normal limits. There was no Bence Jones protein in the urine. The hemoglobin was 12.0 Gm. per 100 ml. and the hematocrit 38 per cent. The sedimentation rate was 33 mm. per hour (Wintrobe corrected). The total serum protein was 7.8 Gm. per 100 ml. with 28 per cent gamma globulin by paper electrophoresis.

X-ray examination of the spine revealed demineralization of spine and pelvis with compression fracture of the twelfth dorsal vertebra.

Sternal marrow aspirate showed a definite increase in plasma cells (10 per cent). Most of these were mature in appearance.

The patient was treated with x-ray to the thoracolumbar spine and urethane was prescribed. She showed symptomatic improvement on this program. The universal reaction was strongly positive.

CASE 3 (B. K.).—This 51-year-old truck driver was referred to University Hospital for treatment of decubitus ulcers. He had undergone a sympathectomy for intermittent claudication one year previously. Postoperatively he developed paralysis and anesthesia below the seventh thoracic segment. He then developed decubitus ulcers. Several grafting procedures were done in another hospital. Because of the poor results, he was referred to this hospital for further care.

Physical examination revealed a paraplegic man in no distress. The blood pressure was 140/96 mm. Hg. The pulse was 96 per minute. There were multiple decubitus ulcers over sacrum and thighs. No pulses were palpable to popliteal space or below, bilaterally.

Urinalysis showed a one plus albuminuria but no Bence Jones protein. There were 5 to 10 white blood cells per high power field in the urinary sediment. The hemoglobin was 9.0 Gm. per 100 ml., the white blood cell count was 16,250/mm.^{3} with 70 per cent neutrophils, 21 per cent small lymphocytes, 6 per cent monocytes, and 3 per cent eosinophils. The platelets were increased in number on the blood film and the red blood cells showed basophilic stippling. The total serum protein was 8.9 Gm. per 100 ml. with 42 per cent gamma globulin on paper electrophoresis. There was no "myeloma peak" on electrophoresis. Howe fraction showed 5.7 Gm. per 100 ml. globulin and 3.2 Gm. per 100 ml. albumin.

Sternal marrow aspirate showed 4 per cent plasmacytes. Some of the plasmacytes were immature in appearance; some were binucleate, and some had 4 nuclei.

X-ray examination revealed diffuse demineralization of bone but no localized bone lesions.

The patient was treated with pedicle grafts and transfusions for a period of 5 months in the hospital. There was no clinical evidence of plasmacytic myeloma during this time. A repeat sternal marrow aspiration done after 2 months in the hospital showed marrow very similar to the first aspirate, except that the plasmacytes were less numerous. Total serum protein 3 months after admission was 7.7 Gm. per 100 ml. with 4.4 Gm. globulin and 3.3 Gm. albumin. The universal reaction was strongly positive.

CASE 4 (T. B.).—This 74-year-old retired man was referred to the University Hospital because of severe anemia. His complaints were referable to the anemia and consisted of weakness and exertional dyspnea of 6 weeks' duration. The anemia had been treated with transfusions.

Physical examination revealed only moderate obesity. The liver and spleen were not enlarged.

Urinalysis showed one plus albuminuria but no Bence Jones protein. The hemoglobin was 10.8 Gm. per 100 ml., red blood cell count 3,800,000/mm.^{3}, hematocrit 33 per cent. The white blood cell count was 3,650/mm.^{3} with 34 per cent segmented neutrophils, 9 per cent band neutrophils, 28 per cent lymphocytes, 6 per cent eosinophils, 1 per cent basophils, and 22 per cent monocytes. There were toxic changes in the monocytes. Sternal marrow aspirate showed normoblastic erythropoiesis. There were toxic changes in the granulocytes and the more immature forms were frequent in number. Plasmacytes were slightly increased in number. Some of these were atypical in appearance with prominent nucleoli. Reticulum cells were increased in number.

The total serum protein was 7.5 Gm. per 100 ml. Paper electrophoresis of the serum protein showed 43.2 per cent albumin, 4.8 per cent alpha-1 globulin, 6.8 per cent alpha-2 globulin, 28.6 per cent beta globulin, and 16.5 per cent gamma globulin.

No bone lesions were demonstrable by x-ray.

The patient was treated with transfusions to maintain his hemoglobin concentration at adequate levels. Universal reaction was strongly positive.

CASE 5 (C. R.).—This 53-year-old farmer was referred to the University Hospital because of a persistent leg ulcer. This had begun 14 months previously after trauma and had failed to heal. During this 14-month period he had had a 28-pound weight loss and marked ease of fatigue.

Physical examination revealed a chronically ill man who was not in acute distress. The liver was palpable 3 cm. below the right costal margin and the spleen 15 cm. below the left costal margin. Small nodes were palpable in the left cervical, right axillary, and both inguinal regions. There was a 4 by 4 cm. ulcer over the right medial malleolus.

Urinalysis revealed 3 plus albuminuria but no Bence Jones protein. The urinary sediment was not atypical.

The hemoglobin was 5.8 Gm. per 100 ml., red blood cell count 2,000,000/mm.^{3} and hematocrit 19 per cent. The white blood cell count was 1,300/mm.^{3} with 6 per cent segmented neutrophils, 7 per cent band neutrophils, 21 per cent monocytes, 15 per cent large lymphocytes, 46 per cent small lymphocytes, and 5 per cent eosinophils. The platelets appeared normal in number on the blood film. There was moderate rouleau formation of the red blood cells.

Sternal marrow was hypocellular. Granulocytic elements showed a toxic change. Erythroid elements were normoblastic. Lymphocytes were relatively increased. A moderate number of plasmacytes were present. Iliac marrow was very similar to sternal marrow. Splenic aspiration showed both mature and immature lymphocytes. There was no evidence of extramedullary hematopoiesis. Left inguinal biopsy showed no abnormality.

The total serum protein was 10.2 Gm. per 100 ml. On paper electrophoresis, this was 24.2 per cent albumin, 6.4 per cent alpha-1 globulin, 9.7 per cent alpha-2 globulin, 18 per cent beta globulin, and 42 per cent gamma globulin.

No bony lesions were demonstrated by x-ray examination of the chest, pelvis, and skull. Skin tests (tuberculin, blastomycin, histoplasmin, and coccidioidin) were all negative. He was thought to have a lymphoma involving spleen and was treated with x-ray therapy to his spleen. Universal reaction was weakly positive.

CASE 6 (O. B.).—This 81-year-old retired man gave a history of recurrent periumbilical pain of 1 year's duration and a history of weakness and ease of fatigue of 2 years' duration. He had been treated for anemia for 5 months before he was referred to University Hospital. This treatment consisted of transfusions, vitamin B_{12}, and iron.

Physical examination revealed a man who appeared much younger than his stated age. The conjunctivae and skin were pale. The liver and spleen were not palpable. Urinalysis was within normal limits. The hemoglobin was 8.1 Gm. per 100 ml., hematocrit 26 per cent, and red blood cell count 2,800,000/mm.^{3} The white blood cell count was 5,300/mm.^{3} with 54 per cent neutrophils, 29 per cent lymphocytes, 13 per cent monocytes, and 4 per cent eosinophils. The monocytes were vacuolated and toxic in appearance. There were 8 per cent reticulocytes. Slight polychromasia of the red blood cells was present. The sternal marrow was cellular with an erythrocyte-granulocyte ratio of 2:5. Plasmacytes constituted 14 per cent of the nucleated marrow cells. Many of these were atypical in appearance.

The total serum protein was 8.0 Gm. per 100 ml. Paper electrophoresis showed 38 per cent albumin, 4.5 per cent alpha-1 globulin, 8.4 per cent alpha-2 globulin, 8.9 per cent beta globulin, and 40.2 per cent gamma globulin. The total serum bilirubin was 1.2 mg. per 100 ml. with 0.1 mg. per 100 ml. direct-reacting bilirubin.

The diagnosis of acquired hemolytic anemia was made. The patient was treated with hydrocortisone and splenectomy was performed. Three months after the operation, the hemoglobin was 13 Gm. per 100 ml. and the hematocrit 42 per cent.

Universal reaction was strongly positive.

DISCUSSION

An outstanding feature of the serologic reaction with lipid antigen is that it behaves like an antigen antibody reaction. In syphilis, for example, the results of serologic tests with lipid antigen and of *Treponema pallidum* immobilization tests, run closely parallel. The main differences lie in the somewhat earlier

appearance of positive reactions with lipid antigen than with treponemal antigen, and in the fact that the treponemal reactions tend to remain longer after antisyphilitic therapy.[6]

It was also observed in this laboratory that the injection of horse serum in rabbits led to an increase in flocculation in their universal reactions over their normal flocculation levels, and this increase corresponded broadly to the appearance of specific precipitins in the blood stream. A tendency was noted for the specific precipitins to appear earlier and disappear sooner than the universal antibodies.[5]

Assuming, therefore, that we are most likely dealing in the universal reaction with an antibody or an antibody-like substance, then the lack of reactivity observed in plasmacytic myeloma corroborates the findings of Lawson and co-workers[1] who reported complete absence or marked deficiency of natural antibodies in this disease. As to the basis of the negative universal reactions in multiple myeloma, it is probably the result of alteration of the globulins, as reported by different authors.

It is likely that this absence of reactivity of the serum of patients with plasmacytic myeloma in the universal serologic reaction may prove an additional aid in the diagnostic armamentarium of this disease. The only other disease known to give negative universal serologic reactions is agammaglobulinemia, which is an extremely rare disease and relatively easy to diagnose.

SUMMARY

The universal serologic reaction was studied in 16 patients with plasmacytic myeloma. Twelve of these gave completely negative reactions and 4 gave unusually weak reactions. It is believed that the absence of or deficiency in reactivity of the universal reaction in this disease is the result of abnormality of the globulins. It is suggested that the universal reaction may offer an additional diagnostic aid in plasmacytic myeloma.

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OBSTETRICAL HEMORRHAGE IN CRIMINAL ABORTION AND ABRUPTIO PLACENTAE

A Study of the Blood Coagulation and Fibrinolytic Systems in Three Cases

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LACK of fibrinogen as a factor in obstetrical hemorrhage has received increasing attention during the past few years. In such conditions as abruptio placentae, intrauterine fetal death, and criminal abortion with attendant uterine trauma, hemorrhage, partly because of a lack of fibrinogen, is a salient feature.[1]

The cause of fibrinogen depletion in these conditions is still a matter for debate. Schneider[2] has shown that placental tissue and its soluble derivatives can initiate intravascular coagulation and the subsequent afibrinogenemia. Amniotic fluid escaping into the general circulation mediates a similar diathesis.[3]

An alternate mechanism thought to underlie the hemorrhage of fibrinogen deficiency is the activation of the fibrinolytic system.[4, 5] In selected cases, low fibrinogen has been correlated with a low profibrinolysin titer and low antifibrinolysin titer, thus suggesting the prior existence of active, effective fibrinolysin levels of short or transient duration.

Clarification of the mechanism of afibrinogenemia is of more than academic interest, inasmuch as effective therapy must be based on an understanding of the pathogenesis. The development and use of a suitable antifibrinolysin would be in order for afibrinogenemia due to fibrinolysin.

On the other hand, afibrinogenemia due to release of thromboplastic materials into the circulation with resultant intravascular coagulation presents a different problem. In this case, the intravenous use of fraction I fibrinogen must be considered. This therapy has met with the disapproval of some investigators because of possible further embolization of fibrin, pulmonary hypertension, and acute shock of the cor-pulmonale type.[6]

This paper describes the blood coagulation findings of 3 cases of obstetrical afibrinogenemia and shows that either mechanism of afibrinogenemia—intravascular coagulation or fibrinolysis—may occur. Further, fraction I fibrinogen has been successfully used as effective therapy for afibrinogenemia caused by the former mechanism, while effective therapy for that caused by the latter mechanism has yet to be developed.

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Received for publication Sept. 16, 1958.

MATERIALS AND METHODS

Venous blood, collected and stored in siliconized glassware with 0.1 volume 3.8 per cent sodium citrate, was used in all studies. Tests were done within 24 hours of collection and the blood was refrigerated when not in use.

Blood Coagulation Studies.—The studies on blood coagulation included the following tests: (1) modified thromboplastin generation tests[7]; (2) one-stage prothrombin time test[9]; (3) modified one-stage tests using 1:1 mixtures of normal plasma and patient plasma, as well as 1:1 alumina-adsorbed normal plasma and patient plasma; (4) factor V level[9]; (5) factor VII level[8]; (6) fibrinogen level[9]; (7) recalcified plasma clotting time[9]; (8) modified plasma clotting time with 1:1 normal and patient plasma mixtures[9]; (9) levels of members of the fibrinolysin system. The initial crude lysin test simply noted the lysis time of clots of recalcified control and patient plasma, or mixtures thereof, at 37° C. Whenever indicated by preliminary studies, more detailed lysin studies were undertaken according to the methods of Ambrus and associates.[10] The results are expressed either in Loomis units or, whenever activities are too low to read quantitatively from the calibration curves, the actual lysis times are given.

CASE HISTORIES

CASE 1.—A 26-year-old Negro woman, primigravida, at term was admitted to the hospital after having been found unconscious at home and breathing stertorously. Clinical diagnosis was abruptio placentae. The patient showed a tendency to bleed and blood samples failed to clot. Bloody amniotic fluid, loss of about 300 ml. of blood per vaginam, and conjunctival hemorrhages were noted. Whole blood and fraction I fibrinogen were administered intravenously. Labor was induced by the intravenous administration of posterior pituitary extract. Delivery was per vaginam. The clinical diagnosis of abruption was confirmed. Except for residual 3-day amnesia, the patient made an uneventful recovery.

CASE 2.—A 36-year-old Negro woman, para ix, gravida x, was diagnosed as a case of abruptio placentae, with afibrinogenemia and intrauterine fetal death. (A Fibrindex test was greater than 10 minutes undiluted.) The whole blood coagulation times were greater than 20 minutes at 2 P.M. and greater than 12 minutes at 2:40 P.M. After administration of 2.1 Gm. of fraction I fibrinogen and 1 unit of whole blood from 3:30 to 4:10 P.M., the whole blood coagulation time was greater than 13 minutes. After further administration of 2 more units of whole blood and 2.7 Gm. of fibrinogen from 4:45 to 6:43 P.M., the whole blood coagulation time was 2 minutes and 15 seconds. The patient then underwent cesarean section. The estimated blood loss was 1,800 ml., representing in the main the retroplacental hematoma; there was minimal blood loss per vaginam. The patient made an uneventful recovery.

CASE 3.—A 36-year-old woman, para iv, gravida vi, in hemorrhagic shock was admitted to a hospital accident ward in the evening with a 3-day history of bleeding per vaginam, becoming profuse immediately prior to admission. The patient, in the sixth month of pregnancy, passed placenta and membranes in the accident ward, but no fetus was found. It was assumed that this was a case of criminal abortion. Blood cultures were not taken. Dilatation and curettage were done to reduce profuse bleeding per vaginam, but the patient died on the operating room table following the administration (within 2½ hours) of 4 units of whole blood, 1 Gm. of fibrinogen, 2 units of whole blood, 2.73 Gm. of fibrinogen, and 2 units of whole blood.

Findings at Postmortem Examination.—There was no definite evidence of previous retroplacental hematoma. A para-ovarian vein hematoma, 1 cm. in diameter, extended from the pelvic brim to the adrenal gland. Both submucosal urethral hemorrhage and labia majora subcutaneous hemorrhage were found. Retroperitoneal ecchymosis, especially in the utero-vesicular space was present. A necrotic area, 2 by 3 cm. in diameter, was noted in the

fundus uteri. Subendocardial hematoma averaging one-half septum, and an epicardial hematoma of left ventricle (possibly due to cardiac massage) was found. Blood was present in trachea and stomach. Petechiae were present throughout the gastrointestinal tract with mucosal and submucosal hemorrhages and hematopericardium. Bilateral pulmonary congestion was present.

RESULTS

Table I shows the results of the coagulation studies of Case 1. The control study done at the same time was that of another patient, primigravida, at term. The first two blood samples show a marked fibrinogenopenia, and a low prothrombin and factor V. These are reflected in the infinite recalcified plasma clotting time and the zero per cent one-stage prothrombin. While bleeding continued, the patient was given whole blood and fibrinogen with immediate improvement of test values. It is of interest that the elevation of fibrinogen levels following the administration of 5.4 Gm. of fraction I fibrinogen was quantitatively ideal. Considering that the blood volume in pregnancy has been found[11] to be increased by as much as 50 per cent (up to 7½ per cent body weight) and that the patient in question weighed 60 kilograms with hematocrit of 0.30, the administration of 5.4 Gm. of fibrinogen should have raised the patient's fibrinogen to approximately 183 mg. per cent. On the other hand, assuming the blood volume to be minimally increased (5 per cent body weight), the fibrinogen level should have been raised to 350 mg. per cent. These values tended to be altered slightly by the subsequent administration of 1 liter of whole blood. The determined value of fibrinogen was 275 mg. per cent. Free fibrinolysin was not found.

Table II shows the results of the coagulation studies of Case 2. The control study was drawn from a patient at term, multipara, and analyzed simultaneously. The patient's first two samples are suggestive of a slow type of coagulation with progressive impairment of the recalcified plasma clotting time, the one-stage prothrombin, and the further diminution of an already low fibrinogen. Administration of fibrinogen and whole blood from 3:30 to 4:10 P.M. effected a definite, although insufficient, improvement. Further administration of whole blood and fibrinogen from 4:45 to 6:30 P.M. resulted in a satisfactory coagulation mechanism, as evidenced by the laboratory tests and the lack of unusual bleeding at cesarean section. It is of interest to note that the elevation of the fibrinogen levels was reasonable if one assumed a blood volume of 7½ per cent body weight (hematocrit 0.30). In this case, the administration of 4.85 Gm. of fibrinogen should have raised the level of the patient's fibrinogen to 210 mg. per cent. On the other hand, an assumed blood volume of 5 per cent body weight leads to a calculation of 280 mg. per cent fibrinogen expected. The value determined was 175 mg. per cent. Again the absence of free fibrinolysin was noted.

The results of Case 3 are presented in Tables III and IV. The sample taken at 2:30 P.M. is of immediate interest. Although the patient had received 4 units of whole blood in the preceding hour, no demonstrable fibrinogen was present since addition of a strong thrombin preparation failed to clot the plasma.

TABLE I

DATA	PATIENT							CONTROL
	10:36* A.M.	11:00 A.M.	12:30 P.M.	12:55 P.M.	1 P.M. TO 1:39 P.M.	1:40 P.M.	2:15 P.M.	10:45 A.M.
R. B. C. (/mm.^{3})	3,490,000			3,100,000		3,120,000	3,140,000	4,333,000
Hemoglobin (Gm. %)	10.3	Blood noted	Conjunctival	9.0	Patient given	9.1	9.0	11.0
Hematocrit	31 %			29 %		27 %	29 %	34 %
W. B. C. (/mm.^{3})	21,600	in amniotic	hemor-	26,600	in this	24,100	36,600	7,940
Bilirubin direct / total	0.16 / 2.33	fluid	rhages		order:			
Platelets (/mm.^{3})	140,000			100,000		85,000	85,000	155,000
Recalcified plasma clotting time	> 1 hr.		noted and	> 1 hr.	2.7 Gm. of	315 min.	265 min.	210 min.
Thromboplastin generation test	74 %		300 ml. of	82 %	fibrinogen,	100 %	100 %	100 %
Prothrombin consumption (serum prothrombin)	40 % (14 %)			23 % (10 %)		64 % (16 %)	44 % (15 %)	85 % (15 %)
One-stage prothrombin	0 % (C. T. > 3 min.)		blood lost	0 % (C. T. > 3 min.)	1 unit of	25 %	34 %	100 %
Factor V	54 %		per vagi-	60 %	blood, 2.7			75 %
Fibrinogen (mg. %)	73			80	Gm. of	275	254	463
Fibrinolysin	Neg.		nam	Neg.	fibrinogen,	Neg.	Neg.	Neg.
Tests done on 1:1 mixture of patient's plasma and alumina-adsorbed normal plasma:					1 unit of			
(a) Recalcified plasma clotting time	5-8 min.			5-8 min.	blood			
(b) One-stage prothrombin	35 %			43 %				

*Time samples were taken.

TABLE II

DATA	PATIENT						CONTROL
	2:48* P.M.	3:28 P.M.	3:30 TO 4:10 P.M.	4:13 P.M.	4:45 TO 6:30 P.M.	6:43 P.M.	3:48 P.M.
Platelets		100,000	Patient given	110,000	Patient given	200,000	170,000
Recalcified plasma clotting time	293 min.	4 hr.	2.1 Gm. of	7·8 min.	2.7 Gm. of	177 min.	194 min.
Thromboplastin generation test	100 %	100 %	fibrinogen	100 %	fibrinogen	100 %	100 %
Prothrombin consumption	53 %	27 %	and 1	60 %	and 2 units	77 %	85 %
One-stage prothrombin	38 %	24 %		46 %		80 %	87 %
Factor V	90 %	80 %	unit of	75 %	of whole	75 %	75 %
Fibrinogen (mg. %)	99	53		116		175	353
Fibrinolysin	Neg.	Neg.	whole blood	Neg.	blood	Neg.	Neg.
Tests done on 1:1 mixture of patients plasma and alumina-adsorbed normal plasma							
(a) One-stage prothrombin	34 %	29 %		31 %		40 %	

Patient underwent cesarean section with minimal bleeding; retroplacental hematoma with estimated 1,400 ml. volume found

*Time samples were taken.

TABLE III

DATA	PATIENT TO 2:30 P.M.*	PATIENT 2:30 P.M.	PATIENT TO 4:10 P.M.	PATIENT TO 4:10 P.M.	PATIENT 4:25 P.M.†	CONTROL NORMAL VALUES
Thromboplastin generation test	Received 4	15 %	Received 4	27 %		100 %
One-stage prothrombin test		0 %	units of	0 %		100 %
One-stage prothrombin of 1:1 patient and normal plasmas	units of	100 %	whole blood	100 %		100 %
One-stage prothrombin of 1:1 patient and normal alumina-adsorbed plasmas	whole blood	0 %	and 3.7 Gm. of	0 %		50 %
Factor V				50 %	5 %	100 %
Factor VII			fraction I	114 % E		40-280 % E
Plasma recalcified clotting time of 1:1 normal and patient plasmas		2 min.	fibrinogen	2 min.	2 min.	2-4 min.
Fibrinogen		None		None		150-350 mg. %
Hematocrit				34 %	31 %	
Crude fibrinolysin: lysis time of 1:1 normal and patient plasmas		15 min.		15-30 min.	30-40 min.	3 days

*Time samples were taken.
†Obtained by intracardiac puncture 3 minutes postmortem.

TABLE IV. MORE DETAILED LYSIN STUDIES

DATA	2:30* P.M.	4:10 P.M.	4:25 P.M.	NORMAL VALUES
Plasmin	Lysis time 24 hours	0.47 μ/ml.	0.49 μ/ml.	No lysis in 72 hours
Plasminogen	30 μ/ml.	1.9 μ/ml.	2.3 μ/ml.	10-30 μ/ml.
Antiplasmin	3.1 μ/ml.	3.5 μ/ml.	3.3 μ/ml.	10-30 μ/ml.
Fibrinolysokinase	Negative	Trace	Trace	Negative

*Time samples were taken.

Under the circumstances, the infinite clotting time in the one-stage prothrombin test is understandable. However, the failure of alumina-adsorbed normal plasma to correct the defect, while unadsorbed normal plasma did correct the defect, implies that the patient's plasma may also have been deficient in prothrombin or factor VII or both. The recalcified clotting time of the mixtures of normal and patient plasma was normal, indicating no strong inhibitor or accelerator activity in the patient's plasma.

The extremely high fibrinolytic activity of the patient's plasma was shown by the 15-minute lysis time of equal mixtures of control and patient plasmas.

The next sample, taken 100 minutes later, presents a generally similar picture. The lack of fibrinogen, the infinite clotting time of the one-stage prothrombin test, and the results with mixtures of patient's plasma and treated normal plasma are striking findings since the patient had received 3 additional

units of whole blood in the interval, and 3.7 Gm. of fibrinogen (fraction I). The lytic potency of the patient's plasma was still extraordinarily high—lysing the clot of an equal mixture of normal and patient plasmas in 15 to 30 minutes.

The last sample, taken by cardiac puncture 3 minutes after the patient was pronounced dead, was grossly hemolyzed, possibly as a result of cardiac massage. Although the relevance of these data may be questionable, it is interesting that the same extremely high fibrinolytic activity was present.

DISCUSSION

Three cases of afibrinogenemia of pregnancy have been studied for defects in blood coagulation. The first two cases were examples of abruption of the placenta. Serial coagulation studies were consistent with the interpretation of intravascular coagulation. The administration of fraction I fibrinogen was effective in both cases both by clinical and by laboratory criteria. Further, there was no evidence that further fibrin formation of any significant degree occurred inasmuch as approximately theoretical levels of fibrinogen were attained in vivo, and the patient's clinical status was markedly improved. Both patients were discharged in good condition.

The third case was a striking contrast to the first two. In this case of criminal abortion, no response was noted either clinically or in laboratory tests, despite heroic measures. The fibrinolysin titer was extremely high—the highest that we have encountered.

It is difficult to avoid the interpretation that the hemorrhagic defect was due, at least in part, to rapid in vivo lysis of blood clotting components in both the patient's own blood and the administered whole blood and plasma fraction. The mechanism of development of this lytic activity is open to speculation.[1, 4, 12] The plasma samples were then frozen in Dry Ice and sent to Roswell Park Memorial Hospital for more detailed lysin studies.

In the first sample of blood obtained from this patient following transfusion of 4 units of whole blood, a trace of free plasmin (too little to measure quantitatively) was present. The plasminogen level was normal (possibly because of the transfusion), but the antiplasmin level was low. No fibrinolysokinase (activator) was demonstrable. Possibly there was a high plasmin and low plasminogen level before transfusion which was subsequently corrected by transfusion; binding of free plasmin by transfused antiplasmin may partly explain the low antiplasmin levels. With time passing and in spite of additional transfusions of blood and fibrinogen, high levels of free plasmin appeared with low levels of plasminogen and antiplasmin. Traces of fibrinolysokinase were demonstrable, too low to quantitate. The low fibrinogen, plasminogen, and antiplasmin levels are in keeping with the high levels of free plasmin. Whether the trace activity of fibrinolysokinase is significant or not can be debated.

Clinically, the fact that fraction I fibrinogen and blood did not meet the needs of the situation is significant. It emphasizes the present lack of suitable, rational therapeutic agents available to the physician. The problem of bleeding due to fibrinolysin is not restricted to the practice of obstetrics. It has been encountered as well in cardiac,[1, 13] pulmonary, abdominal,[14] and other types of

surgery[15, 16]; metastatic prostatic carcinoma[17, 18]; and leukemias.[18-20] Until practical antifibrinolytic agents are developed, treatment of these conditions must remain empirical and unsatisfactory.

SUMMARY

Three cases of afibrinogenemia in pregnancy have been studied for defects in blood coagulation. Two patients having abruption of placenta showed findings compatible with a process of intravascular coagulation as the underlying mechanism of afibrinogenemia. Both cases responded well to intravenous fraction I fibrinogen. The third patient was a case of criminal abortion and had an extraordinarily high fibrinolysin titer. Data are presented to show that the patient's fibrinolytic system was the source of the afibrinogenemia. The heroic administration of fraction I fibrinogen and whole blood did not result in clinical improvement, and the administered proteins were rapidly destroyed by the circulating fibrinolysin. Theoretic and practical aspects of these phenomena are briefly discussed.

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THE CORONARY AND SYSTEMIC HEMODYNAMIC EFFECTS OF SALICYLATE

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IT HAS long been known that body oxygen consumption and carbon dioxide liberation are increased by the administration of salicylate.[1] However, more complete studies of the circulatory effects of this drug were not reported until 1955[2] and 1958[3]; also, the effect of this drug on myocardial metabolism was not included in the preceding studies. It seemed worthwhile, therefore, to repeat observations concerning its general systemic hemodynamic effect along with determination of coronary blood flow and myocardial oxygen metabolism in the intact animal.

METHODS

These studies are reported on 10 intact mongrel dogs varying in weight between 15 and 35 kilograms. The animals were anesthetized with 3 mg./Kg. of morphine intramuscularly followed in 1 hour by 12 mg./Kg. of sodium pentobarbital intravenously. The basic experimental procedure was similar to that previously reported[4] except that mixed venous blood was collected from the pulmonary artery instead of the right ventricle and, in addition to the catheter in the coronary sinus, a third cardiac catheter with its tip in the right atrium was used to record central venous pressure. Approximately 1 hour after the administration of the pentobarbital the cardiac output was determined by the Fick principle and was followed immediately by determination of coronary blood flow by the nitrous oxide saturation method. Pressures were measured by Statham strain gauges and recorded on the Sanborn Poly-Viso with means determined by electrical integration. Expired air was collected in a Tissot spirometer and analyzed with the Scholander apparatus. Blood gas analyses were done by the method of Van Slyke and Neill. When the control studies were completed the dogs were given 100 mg./Kg. of sodium salicylate diluted to make 20 ml. and administered 1 ml. per minute for a 20-minute period. On completion of the injection of the salicylate a second cardiac output was done and this was followed immediately by determination of coronary blood flow.

RESULTS

Results are summarized in Table I. The cardiac rate increased by 10 per cent which was significant at the 0.01 level. Although the mean systemic arterial blood pressure and total peripheral resistance were unchanged there was a consistant and significant increase in pulmonary arterial blood pressure

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This work was supported in part by grants from the National Heart Institute, United States Public Health Service, the Wisconsin Alumni Research Foundation, and the Wisconsin Heart Association.

Received for publication Sept. 22, 1958.

(+17 per cent, $p<0.001$) accompanied by a significant increase in pulmonary vascular resistance (+21 per cent, $p<0.02$). The right atrial mean pressure fell slightly but this was not a significant change and may have been secondary to hyperventilation. Respiratory effects were marked with a 77 per cent increase in minute volume of respiration ($p<0.001$) accompanied by an 87 per cent increase in oxygen consumption ($p<0.001$) and a 90 per cent increase in carbon dioxide liberation ($p<0.001$). The increases in oxygen consumption and carbon dioxide liberation were so similar that the body respiratory quotient remained unchanged. The arterial oxygen content remained the same but there was a considerable decrease in pulmonary arterial oxygen content resulting in an 83 per cent increase in the arteriovenous oxygen difference ($p<0.001$). The coronary sinus oxygen content also decreased significantly with a 12 per cent increase in the arterial coronary sinus oxygen

TABLE I

STUDIES MADE	CONTROL	DRUG	CHANGE	PER CENT CHANGE	S.E.M.*	P <
Cardiac rate per minute	81 ± 17	89 ± 13	+8	+10	2.55	0.01
Mean systemic arterial pressure (mm. Hg)	112 ± 17	114 ± 13	+2	+2	1.89	0.5
Mean pulmonary arterial pressure (mm. Hg)	12 ± 3.1	14 ± 3.7	+2	+17	0.40	0.001
Minute volume respiration (L./min.)	2.6 ± 0.5	4.6 ± 1.2	+2.0	+77	0.30	0.001
O_2 consumption of dog (ml./min.)	102 ± 18	191 ± 31	+89	+87	7.96	0.001
CO_2 liberation of dog (ml./min.)	79 ± 12	149 ± 24	+70	+90	5.89	0.001
A-V O_2 difference (ml./100 ml.)	4.0 ± 0.4	7.3 ± 1.3	+3.3	+83	0.42	0.001
Cardiac output (L./min.)	2.5 ± 0.4	2.7 ± 0.5	+0.2	+8	0.14	0.4
Total peripheral resistance (c.g.s. units)	3,573 ± 636	3,532 ± 718	−41	−1	183	0.9
Total pulmonary resistance (c.g.s. units)	368 ± 99	446 ± 148	+78	+21	25.61	0.02
Left ventricular work (Kg. M./min.)	3.9 ± 0.9	4.2 ± 1.0	+0.3	+8	0.22	0.3
Right ventricular work (Kg. M./min.)	0.4 ± 0.1	0.5 ± 0.1	+0.1	+25	0.03	0.01
Coronary flow (ml./100 Gm./min.)	79 ± 6.3	89 ± 19.4	+10	+13	6.76	0.2
Arterial-coronary sinus O_2 difference (ml./100 ml.)	13.0 ± 1.9	14.5 ± 1.3	+1.5	+12	0.36	0.01
$CMRO_2$ (ml. O_2 cons./100 Gm. myocardium/min.)	10.1 ± 2.8	12.8 ± 2.4	+2.7	+27	1.06	0.05
Index of efficiency (LVW/$CMRO_2$)	0.40 ± 0.09	0.33 ± 0.10	−0.07	−18	0.04	0.2

*Standard error of the mean.

difference ($p<0.001$). The arterial carbon dioxide remained unchanged, whereas the mixed venous carbon dioxide content increased ($p<0.05$) with widening of the mixed venous-arterial carbon dioxide difference ($p<0.01$). There was also an increase in the coronary sinus-arterial carbon dioxide difference ($p<0.01$). The cardiac respiratory quotient remained unchanged. Arterial hemoglobin and hematocrit were unchanged, but the arterial pH rose from 7.29 to 7.31 ($p<0.02$). Cardiac output did not change significantly

nor did the stroke volume. Although the left ventricular work increased slightly this is not statistically significant, but the increase in right ventricle work was significant ($p<0.01$). Coronary blood flow increased 13 per cent, but this increase was so variable that it was not statistically significant ($p<0.1$). The myocardial oxygen consumption per 100 Gm. of left ventricular myocardium per minute however did increase (+27 per cent, $p<0.05$). Coronary vascular resistance and the cardiac efficiency decreased somewhat, but not significantly.

DISCUSSION

Although the observation that salicylate produces an increase in the body oxygen consumption was reported in 1919,[1] clarification of the mechanism of this increase in oxygen consumption was not reported until 1954. Sproull then found an increase in the oxygen consumption of slices of mouse liver incubated in the Warburg apparatus with concentrations of from 3.5×10^{-4} M to 2×10^{-3} M sodium salicylate and pointed out the similarities of salicylate effect to those of 2,4-dinitrophenol.[5] Smith and Jeffrey,[6] in 1956 demonstrated that there was increased oxygen consumption and glycogen breakdown and a decrease in glucose uptake of tissue slices exposed to concentrations of 10^{-3} M salicylate as compared to control tissue slices. In 1956, Brody[7] showed that salicylates in a concentration comparable to toxic levels in the intact animal uncoupled oxidative phosphorylation in liver, kidney, and brain mitochondrial preparations in a manner qualitatively similar to that produced by 2,4-dinitrophenol. Oxygen consumption of mitochondria derived from brain was found to be elevated and fatty acid oxidation in rat liver mitochondria was decreased. Liberation of inorganic phosphate from ATP was enhanced.[7] Experiments in which a tension strain gauge is attached directly to a dog's heart wall are reported by Walton and Darby to show a moderate prompt increase in myocardial contractibility when salicylates are administered. They likened the effect of salicylates to those of 2,4-dinitrophenol and hyperpyrexia.[3] The preceding series of observations suggest that myocardial as well as total body oxygen consumption should be increased by the administration of salicylate and would imply that myocardial efficiency should decrease. This was found in the present in vivo study since myocardial oxygen consumption increased significantly and myocardial efficiency decreased variably (–17.5 per cent), but not statistically significantly ($p<0.2$).

Contrary to the findings of Tenney and Miller,[2] cardiac output did not increase in the present experiments. No ready explanation for this discrepancy is known but it should be stated that the anesthetic was quite different in the two studies. Tenney and Miller used pentobarbital alone (27 mg./Kg. initial dose) and gave a continuous intravenous drip of pentobarbital as a supplement throughout the experiment whereas in this study morphine and pentobarbital were administered each as a single dose and were not supplemented. Pentobarbital anesthesia in the dog tends to cause peripheral vasodilation, tachycardia, and a high resting cardiac output; while morphine and

Nembutal produce a slower rate with sinus arrhythmia. It is possible that these different forms of anesthesia may have influenced the results and explain the difference in cardiac output in the two series. The increase in pulmonary artery pressure in Tenny and Miller's experiments was explained by increased cardiac output. A similar rise in pulmonary artery pressure occurred in our experiments without an increase in cardiac output, but accompanied by an increase in pulmonary vascular resistance. The mechanism for this increase in vascular resistance was not clarified.

The fact that in the present study the myocardial oxygen consumption increased as did the total body oxygen consumption, indicates that the metabolic stimulatory effects of salicylate extend to the myocardium in vivo as well as to muscle slices in vitro. Although there was an increase in the cardiac rate after the administration of salicylate this does not explain completely the increase in myocardial oxygen consumption since the oxygen consumption per beat tended to increase after the administration of salicylate rather than to remain constant as was found previously when cardiac acceleration was produced with an electrical stimulator.[8]

SUMMARY

1. Sodium salicylate intravenously in a dose of 100 mg./Kg. produced in 10 dogs a considerable increase in oxygen consumption and carbon dioxide liberation, accompanied by a similar widening of the arteriovenous oxygen and carbon dioxide difference and no significant change in cardiac output.

2. The pulmonary arterial pressure increased as did the pulmonary vascular resistance.

3. Myocardial oxygen consumption per 100 Gm. of left ventricular myocardium per minute increased significantly, but left ventricular work did not.

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QUANTITATIVE MEASUREMENT OF GASTROINTESTINAL BLOOD LOSS

II. Determination of 24-Hour Fecal Blood Loss by a Chemical Photospectrometric Technique

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PREVIOUS workers[2-8] have described the quantitation of the 24-hour fecal blood excretion by means of the radioisotopes sodium chromate[51] and iron[59]. Although the sodium chromate[51] technique is quite accurate and reasonably simple to perform, it would not be suitable for large scale routine screening because of the time involved for the performance of an individual determination, the expense, and the undesirability of administering radioactive materials on a routine basis to a large number of patients. For this reason a study was undertaken to develop a chemical test for determining quantitatively the normal range of 24-hour fecal hemoglobin excretion in human beings.

METHODS AND MATERIALS

Homogenization of Feces.—Feces were collected either directly into a paper quart container by the patient, or removed from the bedpan into the quart container. The feces were placed in a weighed quart-sized blender,* and the weight of the 24-hour stool plus blender determined. Then 3 to 4 parts, by weight, of distilled water were added to the blender, the exact weight determined, and the feces homogenized for two minutes. The qualitative benzidine dihydrochloride-H_2O_2 occult blood test was done on the feces and the homogenate. If the qualitative test was negative for occult blood, 10 Gm. of the homogenate was added to a weighed 250 Erlenmeyer flask and the exact weight of the amount of homogenate determined. Then about 90 ml. of water was added to the flask and the weight again determined. This would represent an approximate dilution of aqueous solution of feces of 1:40 to 1:50. If the qualitative test was strongly positive, then 1 or 2 Gm. of homogenate only was added to the weighed Erlenmeyer flask followed by 100 ml. of water. This represented an aqueous fecal suspension of 1:200 to 1:500.

Quantitative Benzidine Base Technique for 24-Hour Fecal Hemoglobin.—The benzidine base test described by Crosby and associates[9] for quantitating plasma hemoglobin has been adapted for use in this study.

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Supported by Grant A-1149, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda 14, Md.
Presented by title before the American Society of Clinical Investigation, June, 1958.[1]
Received for publication Sept. 22, 1958.

*A Waring Blendor was used throughout.

Reagents.—One gram per cent of benzidine solution base was made by dissolving 1 Gm. of benzidine base in 90 ml. of glacial acetic acid, plus 10 ml. of distilled water. The reagent was kept in a dark screw-top bottle in the refrigerator and was renewed weekly.

One per cent hydrogen peroxide was made by diluting 30 per cent stock H_2O_2, 1:30 with distilled water. Fresh solution was made daily.

The hemoglobin solution was made as follows: 10 ml. of red cells were washed twice with an equal volume of 0.9 Gm. per cent sodium chloride and sufficient saline added to make a final concentration of approximately 10 Gm. per cent of hemoglobin. The cells were then lysed by freezing and thawing twice in a Dry Ice and alcohol bath; then, 1 ml. amounts of the concentrated hemoglobin solution were placed in a series of small tubes which were kept frozen; one such tube was thawed for one week of use. On the day of the test, the concentration of the hemoglobin solution was determined by the cyanmethemoglobin technique. The hemoglobin was determined each day tests were run because of the slight amount of evaporation which occurred during a week's time, causing a progressive rise in the concentration of hemoglobin.

The hemoglobin standard was made up in duplicate by adding 0.02 ml. of the concentrated hemoglobin solution to 10 ml. of saline. If the concentrated hemoglobin solution was 10 Gm. per cent this would mean that the hemoglobin standard had a concentration of 20 mg. per cent.

Ten per cent glacial acetic acid was used as a diluent.

All glassware is given a final wash in dilute nitric acid, rinsed several times in distilled water, and dried by air. The importance of this step can not be overemphasized, since its omission will often lead to erratic results.

Sulfate-free distilled water was obtained from a resin column.*

Procedure.—One milliliter of benzidine base solution was added to a 15 ml. test tube followed by 0.02 ml. of the aqueous fecal suspension. This was followed by 1 ml. of 1 per cent hydrogen peroxide. The contents were mixed by tapping or swirling the tube. After 20 minutes the color change was complete; 10 ml. of 10 per cent acetic acid was added and the contents mixed by inversion. Ten minutes later the contents were transferred to matched Klett tubes and read in the Klett photometer with the 520 mμ filter.

Duplicate determinations were done on all unknowns. A reagent blank and two hemoglobin standards were run in duplicate with each day's run.

Sample Calculation.—Fecal weight per 24 hours 80.9 Gm.; feces plus H_2O in blender 290.0 Gm.; 10.10 Gm. of 1:4 fecal homogenate added to Erlenmeyer flask plus 100.15 Gm. of H_2O for a total weight of 110.25. Average Klett readings were: blank, 18; 25.2 mg. per cent hemoglobin standard, 183; and unknown 122. Hemoglobin concentration of unknown $= \frac{122 - 18}{183 - 18} \times 25.2 = 15.9$ mg. per cent; hemoglobin in entire fecal sample $= \frac{110.25}{100 \times 10.10} \times 15.9 \times 290.9 = 504$ mg. Circulating hemoglobin of the patient $= 8.2$ Gm. per cent, so $\frac{504}{82 \times 1}$ or 6.2 ml. of blood was lost per day.

Preliminary experiments demonstrated that more than 90 per cent of the hemoglobin was present in the fecal particles of the fecal homogenate. Attempts to extract the hemoglobin from the fecal particles with acid acetone (2 parts to 1 part of 1:4 aqueous fecal suspension) resulted in erratic extraction of from 15 to 100 per cent of the hemoglobin in the supernatant. Further attempts to explore other agents for extraction were abandoned when satisfactory recoveries were obtained by using whole 1:40 to 1:200 aqueous dilution of feces. Although some free particles are present, they could not be seen in the test tubes after a further 500 fold dilution of the 1:40 to 1:200 aqueous fecal suspension with the benzidine, H_2O_2, and 10 per cent acetic acid reagents.

Feces were stored at 4 to 6° C. for as long as 3 weeks before testing. The fecal hemoglobin-benzidine reaction remained constant for one 1:40 aqueous fecal suspension for 39

*Bantum demineralizer and sterilizer, Barnstead Still Corporation.

days at 4 to 6° C. (Values of 35, 36, 37, 30, 32, and 26 mg. per cent of hemoglobin were obtained for 18, 29, 39, 48, 65, 84, 85, and 89 days of storage, respectively.)

Radioactive Chromium^{51} *Determination of the Quantitative 24-Hour Fecal Blood Excretion.*—This technique has been described in detail in a previous communication.[5] In brief, the technique consisted of removing an aliquot of blood from the subject, tagging it in vitro with sodium chromate, and reinjecting the blood back into the donor. The counts per minute per milliliter of circulating whole blood during the time of fecal collection as well as the total counts per minute which were excreted in a 24-hour fecal sample were then determined. The ratio of the total counts per minute in the 24-hour fecal sample and the counts per minute per milliliter of circulating whole blood gave the apparent whole blood fecal excretion in milliliters.

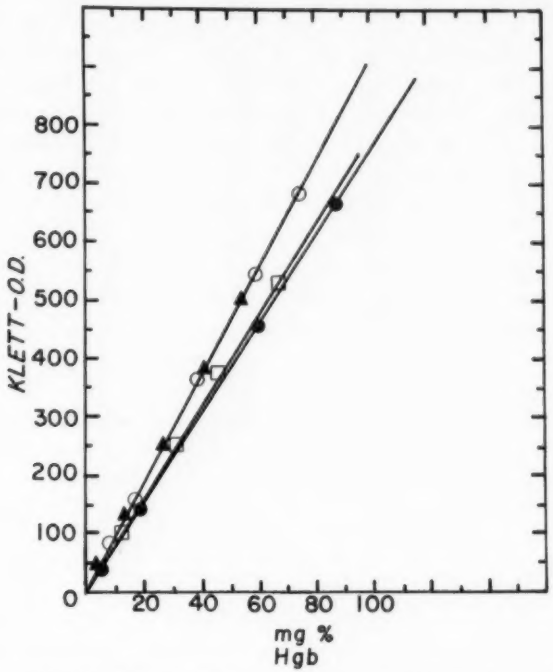

Fig. 1.—The relationship between Klett readings and hemoglobin concentration in aqueous fecal suspensions. Each symbol represents a different day's run.

RESULTS

Influence of Fecal Concentration.—Sufficient hemoglobin was added to 200 to 500 ml. of 1:4 aqueous fecal homogenate to make the final concentration of hemoglobin about 6 mg. per gram. Aliquots of the 1:4 fecal homogenate were then further diluted with distilled water to make the final concentration of feces and water from 1:36 to 1:200. In Fig. 1 it can be seen the concentration of hemoglobin of such fecal samples varied linearly with the Klett readings of from 0 to 600. The degree of aqueous dilution of feces between the values 1:36 to 1:200 did not affect the optical density of the benzidine-hemoglobin solution

(Fig. 1). A straight line was obtained for a series of fecal dilutions ranging from 1:36 to 1:200 for each experiment. The slope of the line varied from day to day (Fig. 1), therefore, it was necessary to run a hemoglobin standard with each day's determinations.

TABLE I. FECAL BLOOD (MEN)

SUBJECT	CLINICAL STATUS	AGE	COLLECTION INTERVAL (HOURS)	WEIGHT OF ENTIRE STOOL (GRAMS)	BLOOD LOST PER DAY (ML.)
1	Normal	11	24	126.0	7.0
2	Paraplegic	19	48	86.1	2.6
3	Normal	20	24	82.2	1.1
4	Normal	20	24	147.3	8.6*
5	Normal	21	24	81.1	2.3
6	Normal	21	24	72.3	0.8
7	Normal	21	24	108.9	8.9*
8	Normal	21	24	205.8	3.4
9	Normal	21	24	132.1	0.3
10	Normal	21	24	143.6	1.1
11	Normal	21	24	128.6	1.4
12	Normal	21	24	178.3	1.4
13	Normal	21	24	203.3	3.1
14	Normal	21	24	69.4	4.2
15	Normal	21	24	42.9	1.0
16	Normal	22	24	64.3	0.9
17	Normal	22	24	132.0	1.4
18	Normal	22	24	210.1	1.6
19	Normal	22	24	27.1	0.8
20	Normal	22	48	200.0	1.6
21	Normal	22	48	157.5	0.9
22	Normal	22	24	104.4	3.5
23	Normal	22	24	237.1	3.1
24	Normal	22	24	63.8	1.1
25	Normal	22	24	38.0	1.9
26	Normal	22	24	72.9	2.3
27	Normal	22	24	114.3	1.3
28	Normal	22	24	96.3	11.4*
29	Normal	22	24	233.7	5.4
30	Normal	22	24	216.5	1.2
31	Normal	22	24	28.4	2.4
32	Normal	22	24	111.1	1.9
33	Normal	22	24	124.1	2.5
34	Normal	22	24	53.8	1.8
35	Normal	22	24	56.1	0.0
36	Normal	23	24	80.2	2.4
37	Normal	24	24	49.9	1.0
38	Normal	24	24	93.4	0.9
39	Normal	26	24	226.4	1.3
40	Normal	27	24	105.9	3.1
41	Normal	27	24	144.7	3.0
42	Normal	27	24	148.8	1.3
43	Normal	30	24	117.7	0.8
44	Normal	32	24	97.2	1.7
45	Normal	35	24	62.2	0.7
46	Hand injury	39	24	47.0	0.5
47	Mitral stenosis	39	24	277.0	3.1
48	Normal	39	24	132.6	1.3
49	Spinal fusion	45	24	217.3	2.0
50	Emphysema	63	24	382.0	2.7
51	Mitral stenosis	66	48	90.1	2.1
52	Osteoarthritis	73	24	31.2	1.8
		Mean ± S.D.		2.38 ± 2.28	
		Adjusted mean ± S.D.		1.94 ± 1.29	

*Eliminated in calculation of adjusted mean because they were at least 2.0 S.D. greater than the mean.

In Vitro Recovery of Added Hemoglobin.—The fecal hemoglobin was determined on a 1:40 aqueous fecal suspension. Then a known amount of hemoglobin was added to this suspension and the hemoglobin concentration again determined. The difference between the two chemical determinations was then compared with the known amount of hemoglobin added and expressed as per cent recovery. In 64 experiments an average recovery of 101 ± a standard deviation (S.D.) of 20 per cent was observed,* with a range of from 73 to 145 per cent.

In Vivo Recovery Experiments.—Twelve normal subjects were fed 80 ml. of chromium^{51}-labeled whole blood, in ACD solution,† and the stools collected for 7 days after drinking the blood. An average of 105 ± 10 per cent (89 to 119) was recovered by the chromium^{51} technique compared to a recovery of the same fecal samples of 106 ± 13 per cent (85 to 120) by the benzidine quantitative technique. In a second experiment in which 11 normal subjects were fed 80 ml. of blood, an average of 90 ± 7 per cent (76 to 113) was recovered in the 7-day stools by the chromium^{51} technique compared to an average recovery of 111 ± 10 per cent (92 to 124) by the benzidine technique.

Normal Fecal Blood Excretion.—In Table I are listed the age, clinical status, weight of feces, and milliliter of whole blood excreted per day in the fecal sample for 52 males, and in Table II for 24 females. None of the presumed normal subjects had gastrointestinal x-rays or proctoscopy, but all had normal peripheral blood counts and did not present signs or symptoms of alimentary canal disease.

It can be seen that the predominant age group studied was between 20 and 30 years old; most of the male subjects were medical students or physicians, and most of the female subjects were nurses. In cases where more than one 24-hour fecal collection was made, the first collection only was used in order to avoid bias in the statistical study. The fecal whole blood loss was determined by dividing the milligrams of hemoglobin in the fecal sample by the milligrams of hemoglobin per milliliter of circulating blood at the time of study. In instances where the subject had not passed a fecal sample 24 hours before the collection of the fecal sample, the milliliters of blood present in the entire fecal sample was divided by the number of days elapsing since passage of the preceding fecal sample.

Among the men there were 3 individuals (Subjects 4, 7, and 28, Table I) who excreted amounts of blood which were more than 20 S.D. greater than the mean. Among the normal women studied, Subjects 8 and 22 (Table II) exceeded the mean by more than 2 S.D. and were eliminated in calculating the adjusted mean (Table II). Normal values for the men and women are recalculated omitting these individuals and are presented as the adjusted means in Tables I and II.

*Hereafter, variance of all means will be expressed as ± 1 standard deviation.
†Acid citrate dextrose solution, NIH formula B, 100 ml. per 500 ml. of blood.

A significant difference* ($p < 0.05$) existed between the milliliter per day of blood excreted by all the men and all the women (Table II). The men also excreted significantly more blood ($p < 0.02$) than the women when the adjusted means are used (Tables I and II). If the adjusted mean for men and women of 1.7 ml. of blood for 24 hours is used, then the normal range of fecal hemoglobin excretion based on ± 2 S.D. (95 per cent of all normal values) would be 0 to 4.2, or based on the mean ± 3 S.D. (99.5 per cent of all normal values) 0 to 5.4 ml. of blood.

TABLE II. FECAL BLOOD (WOMEN)

SUBJECT	CLINICAL STATUS	AGE	COLLECTION INTERVAL (HOURS)	WEIGHT OF ENTIRE STOOL (GRAMS)	BLOOD LOST PER DAY (ML.)
1	Normal	2	24	58.5	0.3
2	Normal	3	24	45.3	0.8
3	Normal	4	48	177.0	1.0
4	Normal	9	24	47.4	0.7
5	Normal	11	24	62.3	3.6
6	Normal	17	24	133.0	2.1
7	Normal	18	24	65.7	0.0
8	Normal	18	24	85.7	4.0*
9	Normal	18	24	53.6	2.6
10	Normal	18	24	77.7	0.5
11	Normal	19	24	43.9	2.2
12	Normal	19	24	127.6	0.8
13	Normal	19	24	40.5	2.2
14	Normal	20	24	100.8	1.5
15	Normal	22	48	114	0.4
16	Normal	35	24	72.3	1.1
17	Reactive depression	26	24	31.1	0.3
18	Normal	36	24	47.5	0.4
19	Hypertensive	51	24	96.1	0.6
20	Leg ulcer	52	72	813.5	1.7
21	Leg amputation	60	24	123.1	0.6
22	Broken hip	65	24	35	3.9*
23	Arteriosclerotic heart disease	73	24	118.6	0.6
24	Broken hip	74	72	144.4	1.2
	Mean ± S.D.			1.38 ± 1.17†	
	Adjusted mean ± S.D.			1.14 ± 0.90‡	
	Adjusted mean ± S.D. for men and women			1.69 ± 1.24	

*Eliminated in calculation of adjusted mean because they were at least 2.0 S.D. greater than the mean.
†Significantly smaller than the mean for men ($p < 0.05$).
‡Significantly smaller than the mean for men ($p < 0.02$).

Forty-eight additional stool specimens were collected from some of the normal men. Serial samples of 4 or more from one individual are summarized in Table III. On one occasion, 6 men exceeded 5.4 ml. of fecal blood for 24 hours (Subjects 1, 4, 7, and 28, Table I; Subject 45, Table III) plus one duplicate sample of Subject 18 containing 7.2 ml. of blood. Thus 6 out of 100 fecal

*Statistical formula[10]:

$$\text{Standard deviation (S.D.)} = \sqrt{\frac{\Sigma X^2}{n-1} - \frac{n}{n-1}\Sigma\overline{X}^2}$$

$$\text{Standard error (S.E.)} = \frac{\text{S.D.}}{\sqrt{n}}$$

$$\text{S.E. difference of means} = \sqrt{\text{S.E.}_1^2 + \text{S.E.}_2^2}$$

$$t = \frac{\overline{X}_1 - \overline{X}_2}{\text{S.E.}_{1\text{-}2}}$$

$\overline{X}$ = mean.

samples or 6 per cent could be considered as false positive results if the assumption was made that none of these individuals had gastrointestinal bleeding. An additional 23 multiple samples on the women (Table III, for serial samples of 4 or more) were measured giving a total number of fecal specimens on 24 women of 47. Two women, Subject 18 (Table III) and a duplicate value on Subject 12 (Table I) of 10.8 ml., exceeded 5.4 ml., thus giving a potential incidence of false positives of 4 per cent in the women. The dietary history of the presumed normal individuals with increased amounts of fecal hemoglobin excretion on one occasion were no different from the rest of the subjects in this series with respect to ingestion of large amounts of raw red meat or liver. Subject 45 (Table III) ingested one pound of rare beefsteak on Oct. 15, 1957, with no significant increase in the fecal blood excretion of the next fecal sample passed two days later (Table III).

The Relative Sensitivity of the Benzidine Quantitative Test for Detecting Hemin and Hemoglobin.—Inasmuch as the structure of the hemoglobin molecule might be altered by intestinal enzymes and bacteria, the ability of hemin alone to react with benzidine base was determined in 14 experiments. Hemin* solutions varying in concentrations of from 0.1 to 2.06 mg. per cent and representing the concentration of heme in from 2.6 to 53 mg. per cent solutions of hemoglobin (milligram of hemin ÷ 0.0386) resulted in 78 ± 9.1 per cent as much color development as would be expected from the calculated hemoglobin equivalent.

Effect of Foods and Drugs.—In order to check the possible effect of various commonly ingested foods, representative foods shown in Table IV were added directly to the blender and homogenized by the addition of 4 parts of water and further diluted to 1:40 with water. The results are expressed as milligrams per cent of hemoglobin. Ferrous sulfate, 0.6 Gm., gave a negligible hemoglobin equivalent effect (Table IV). Since it is known that the sulfate ion inhibits the benzidine base reaction,[9] the hemoglobin equivalent of ferric chloride, 1.1 Gm., was also measured and found to be 1.1 mg., a value not significantly different from 0.6 Gm. of ferrous sulfate which contains approximately the same amount of iron. One hundred grams of raw hamburger had the equivalent of 399 mg. of hemoglobin, or approximately 2.5 ml. of whole blood. Cooking the hamburger reduced the values approximately one half (Table IV). Of all the foods studied raw liver caused the greatest color reaction; methantheline bromide (Banthine) and a mixture of magnesium and aluminum hydroxide (Maalox), drugs commonly used in ulcer therapy, had no significant effect on the test (Table IV).

Effect of Oral Iron.—A 63-year-old man with a history of a bleeding duodenal ulcer and confirmatory x-ray findings was admitted to the hospital because of epigastric pain. The patient was placed on a bland diet with Maalox for one week and then ferrous sulfate, 0.3 Gm. t.i.d., orally because of an anemia of 11 Gm. per cent hemoglobin and presumption of blood loss in the

*Obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio.

past. Eighteen consecutive 24-hour fecal collections were made and the patient then kept on the same dietary regime without iron for 20 more consecutive 24-hour fecal samples. The patient's red cells were tagged with Cr^{51} and the 24-hour fecal blood loss was simultaneously determined by the Cr^{51} technique and the chemical benzidine technique.

TABLE III. MULTIPLE FECAL SAMPLES

SUBJECT	AGE AND SEX	DATE	FECAL WEIGHT (GRAMS)	COLLECTION PERIOD (HOURS)	WHOLE BLOOD (ML.)
45	35, M	6/10/57	62.2	24	0.7
		6/13/57	200.0	24	0.7
		7/24/57	193.1	48	2.7
		7/30/57	81.2	48	0.6
		9/23/57	117.7	24	1.4
		10/ 1/57	117.7	24	1.4
		10/ 3/57	115.7	24	0.5
		10/ 4/57	154.3	24	9.8
		10/ 5/57	198.2	24	3.4
		10/17/57	324.2	96	1.7
		10/21/57	178.7	96	0.7
		10/22/57	115	24	2.5
		10/23/57	143.8	24	5.1
		10/24/57	155.8	24	3.9
		11/27/57	273.0	48	2.6
		11/29/57	231.2	48	1.7
		12/ 1/57	133.7	48	1.3
		12/ 6/57	217	72	2.0
		12/ 8/57	216.7	48	1.5
40	27, M	6/21/57	106	24	3.1
		7/26/57	104.5	48	5.0
		10/ 9/57	101.8	24	3.1
		10/10/57	81.0	24	3.2
		10/16/57	80.8	24	1.4
		10/17/57	84.7	24	2.4
		10/19/57	102.8	24	3.5
		8/11/57	160.6	48	0.7
		8/13/57	59.4	48	0.5
		8/15/57	85.5	48	0.9
36	23, M	10/ 9/57	80.2	24	2.4
		10/11/57	35.8	24	1.5
		10/15/57	125.1	24	2.7
		10/16/57	176.6	24	1.8
18	36, F	10/18/57	47.5	24	0.4
		10/21/57	49.0	24	5.9
		10/21/57	50.0	24	3.7
		10/25/57	69.7	24	1.5
		10/27/57	53.6	24	1.3
		11/29/57	63.8	48	1.0
15	22, F	11/21/57	114	48	0.4
		11/26/57	66.3	48	0.7
		12/ 5/57	83.7	24	1.6
		12/ 7/57	155.3	48	0.7
1	2, F	10/16/57	58.5	24	0.3
		10/17/57	26.4	24	0.4
		10/18/57	31	24	0.0
		10/19/57	41	24	0.3
		12/10/57	113.0	24	0.9
		12/12/57	80.8	24	0.7
		12/16/57	163.5	48	0.9
		12/18/57	75.6	24	0.2
		1/ 7/58	125.2	48	1.1
		1/ 9/58	105.1	24	0.5

The mean fecal blood loss as measured by Cr^{51} and the benzidine techniques and the mean daily difference between the two during and after iron therapy are summarized in Table V. Iron therapy did not increase the fecal blood loss as measured by Cr^{51} but did cause a significant increase in the apparent fecal blood loss as measured by benzidine (Table V). The figure labeled "Benzidine minus Cr^{51}" in Table VI is the mean of the difference between the benzidine and the Cr^{51} results in each fecal sample.

TABLE IV. MILLIGRAMS OF HEMOGLOBIN EQUIVALENT OF SOME REPRESENTATIVE FOODS

FOOD	AMOUNT (GRAMS)	HEMOGLOBIN EQUIVALENT (MILLIGRAMS)
Egg	47	0
Milk	101	1.5
$FeSO_4$	0.6	1.6
$FeCl_3 \cdot 6H_2O$	1.1	1.4
Hamburger, raw	100	399
Hamburger, cooked	101	188
Ice cream	98	43
Pork, raw	49	134
Haddock, raw	40	31
Lamb, raw	50	255
Liver, raw	100	612
Turkey, cooked	89	53
Banthine	1	20
Maalox	100	4.6

TABLE V. EFFECT OF ORAL IRON

Ferrous Sulfate (0.9 Gm. per day)	
Cr^{51}	0.8 ± 0.35 ml. blood per day
Benzidine	3.1 ± 1.44
Benzidine minus Cr^{51}	2.3 ± 1.45
	N = 18
No Ferrous Sulfate	
Cr^{51}	0.9 ± 0.31 ml. blood per day
Benzidine	2.1 ± 1.42*
Benzidine minus Cr^{51}	1.2 ± 1.37†
	N = 20

*Statistically significantly less than when patient on iron ($p < 0.05$).
†Statistically significantly less than when patient on iron ($p < 0.02$).

A normal 21-year-old woman had an average fecal blood loss determined by the benzidine technique of 0.78 ml. per day ± 0.66 for ten 24-hour fecal samples. The subject was then placed on ferrous sulfate 0.3 Gm. t.i.d., and the mean of six 24-hour stools was 1.57 ± 0.64 ml. per day. The difference of these two means is significant ($p < 0.05$).

Comparison of the Benzidine Technique With the Chromium^{51} Technique in Bleeding Patients.—In Table VI are listed the milliliter of fecal blood per day recovered from a patient with hereditary telangiectasia who was losing substantial amounts of blood from his gastrointestinal tract. Over a 7-day period a total of 213 ml. of blood were recovered by the Cr^{51} technique and a total of 247 by the benzidine technique, a value of 16 per cent higher than that determined by Cr^{51}.

Fifty-seven patients who were suspected of having gastrointestinal bleeding lesions were studied simultaneously by Cr^{51} and the benzidine technique with at least one 24-hour fecal sample. All were in the normal range of from 0 to 5.4 ml. of blood per day by the Cr^{51} test. All but 2 of these 57 patients were also within normal limits by the benzidine test; the 2 exceptions being values of 5.9 and 6.0 per day by the benzidine test compared to 1.4 and 0.4 ml. as measured by Cr^{51}.

TABLE VI. COMPARISON OF BENZIDINE AND Cr^{51} IN BLEEDING PATIENTS

MILLILITER OF FECAL BLOOD PER DAY	
Cr^{51}	BENZIDINE
51	67
25	33
8	10
21	34
34	39
45	50
29	14
213	247 (+16%)

Fecal Blood Quantitation in Randomly Selected Patients With Known Gastrointestinal Bleeding Lesions.—Six patients are shown in Table VII in whom random 24-hour stool samples were collected and quantitative fecal hemoglobin excretion determined.

TABLE VII

AGE	SEX	DIAGNOSIS	FECAL BLOOD (ML. PER DAY)
35	F	Iron deficiency	35, 10, 34, 88
57	M	Hereditary telangiectasia	25, 33, 18, 47, 13, 21, 51, 138
70	F	Hereditary telangiectasia	8, 70
68	M	Carcinoma of colon	5, 10
61	M	Myeloma	7, 11, 12, 14, 17
4	M	Chronic GI blood loss	3, 5
83	M	Duodenal ulcer	4, 23, 44

DISCUSSION

The average fecal blood loss for men and women in this study is 1.7 ml. per day, a figure 31 to 41 per cent higher than the values of Roche and associates[2] of 1.3 ± 0.6 and Ebaugh and co-workers[5] of 1.2 ± 0.5 ml. of whole blood per day measured by the Cr^{51} technique. In the current study simultaneous determination of fecal blood loss by Cr^{51} and benzidine technique resulted in fecal blood excretions 12 to 16 per cent higher as measured by benzidine than by Cr^{51} in bleeding patients and volunteers who drank blood.

Certain individuals with fecal blood excretions in the normal range showed a persistent elevation by the benzidine technique as compared to the Cr^{51} technique, which were as much as 230 per cent greater than the Cr^{51}. However, although the difference is statistically significant and large percentage-wise, it actually amounted to only 1.2 ml. of blood per day (Table V). The difference between the Cr^{51} and benzidine was consistently much less in

feces from patients with increased fecal blood excretion (16 per cent higher, Table VI, and 12 per cent in the experiments on patients given oral blood). The reason for these differences in fecal blood recovery between the Cr^{51} and benzidine technique was not determined in this study. Actually the difference between the two techniques is of little practical consequence as far as using the benzidine test as a screening test, since only 2 out of 56 fecal samples (4 per cent) found by the Cr^{51} to be in the normal range were outside the normal range when assayed by the quantitative benzidine base technique.

It would not appear necessary to place the patients on a special diet when employing the quantitative benzidine test as a screening procedure for detecting abnormal gastrointestinal bleeding, since with the exception of raw red meat and raw liver other dietary constituents tested in vitro had little effect on the benzidine test. Perhaps the initial dilution of feces, 1:40 with water, plays a role in minimizing the reaction of nonhemoglobin fecal constituents with benzidine base.

Oral iron therapy does significantly increase the amount of apparent gastrointestinal blood loss as measured by benzidine by about 1 ml. per day. This effect of oral iron cannot be explained by actual increase in loss of blood in the feces since the Cr^{51} fecal blood excretion did not increase in the same patient while on iron therapy. The implication from the above results, that iron may have a direct effect on benzidine, was not borne out by in vitro studies, so that the in vivo effect of iron found in this study remains unexplained.

The small but significant increase in gastrointestinal blood loss in men, as compared to that of women, is of interest. Men lose about 24 ml. more blood in the feces per month than do women. Thus, men do not have quite as much advantage over women with respect to iron loss as was formerly thought. The average menstrual blood loss is about 50 ml. a month or 25 mg. of iron. Men lose about 12 mg. more of iron per month in their feces than do women, so that the favorable iron balance in men compared to women is closer to 13 mg. rather than 25 mg. a month according to the data in this study. The above discussion is all predicated on the assumption that the benzidine measures proportionally the same amount of nonhemoglobin substances in the feces in men and women. Since no attempt was made in this study to match the men and the women with respect to weight and surface area, the possibility that the difference in fecal hemoglobin excretion between the sexes is due to the average lower weight and hence smaller gut surface area of the women in comparison to the men in the series cannot be excluded. Further study is indicated of the fecal hemoglobin excretion in a series of men and women matched for weight and surface area since the results in this study could also be explained by the known higher instance of peptic ulceration in the men.

SUMMARY

1. A simple and reasonably precise chemical method employing the benzidine base-H_2O_2 reaction for measuring 24-hour fecal hemoglobin excretion has been described.

2. Forty-nine men were found to shed 1.9 ± 1.3 ml. (1 standard deviation) of whole blood per day in the feces and 22 women 1.2 ± 1.0 ml. per day. Men showed a significantly greater excretion of blood in the feces than women ($p < 0.02$).

3. The quantitative benzidine test resulted in a 12 to 41 per cent higher apparent fecal blood loss as measured by the quantitative benzidine test.

4. Oral ferrous sulfate caused a significant increase (about 1 ml.) in apparent fecal blood loss as measured by the quantitative benzidine test.

5. All the data in this study was obtained on subjects on ad libitum diets. It would appear feasible to employ this test as a screening procedure without imposing dietary restrictions.

We would like to acknowledge the help of the members of the classes of 1956, 1957, 1958, and 1959 of Dartmouth Medical School who served as volunteer subjects for some of these studies.

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LABORATORY METHODS

THE DETECTION OF DEFECTS OF FIRST STAGE OF COAGULATION

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THE description of the so-called thromboplastin generation test (TGT)[1] constituted an important contribution to the understanding of the mechanism of the coagulation of blood and especially of the phenomena which precede the activation of prothrombin to thrombin. This technique is relatively simple and has had large acceptance. However, many question the significance of the results obtained with it, maintaining that they are related only partially to thromboplastic activity. Besides the difficulties of interpretation, the precision and standardization of the thromboplastin generation test are criticized because reliance is placed on materials obtained from normal subjects rather than on mixtures of purified substances of measurable quantities to which unknowns may be added. The study of platelets is especially precarious because of the difficulty in their measurement. The purpose of the present paper is to describe the technique used in our laboratory to perform the thromboplastin generation test and its application and interpretation.

TABLE I

TERMS USED IN THIS ARTICLE	TERMS CONSIDERED AS POSSIBLE SYNONYMS
Platelet thromboplastic factor or activity	Platelet factor 3
Antihemophilic factor (AHF)	Antihemophilic globulin (AHG), thromboplastinogen, platelet cofactor I, factor VIII
Plasma thromboplastin component (PTC)	Christmas factor, platelet cofactor II, factor IX, autoprothrombin II
Plasma thromboplastin antecedent (PTA)	
Plasma thromboplastin	Thrombokinase
Tissue thromboplastin	Thromboplastin
Factor V	Plasma Ac-globulin, labile factor, proaccelerin
Stable factors (serum prothrombin conversion factor [SPCA] and Stuart factor)	Factor VII, proconvertin, Prower factor, cothromboplastin, autoprothrombin I*

*In the light of the present knowledge, it is impossible to ascertain from the literature which of these factors are identical with SPCA or with the Stuart factor. Comparative studies of these factors have been recently made by Hougie and associates[2] and Graham and co-workers.[3]

Explanation of Terms.—Table I presents a list of terms used in this paper and some of their synonyms.

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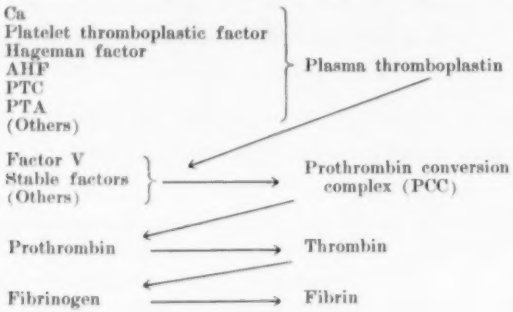
This study was assisted by a grant from the Michigan Alumni Fund of the Horace H. Rackham School of Graduate Studies and by a grant from the Michigan Heart Association.

Received for publication July 31, 1958.

The following scheme of the succession of reactions which take place during the coagulation of blood is presented as a simplified explanation and working hypothesis. It is recognized that this is an artificial representation of the mechanism of clotting which is, as yet, incompletely understood.



MATERIALS AND METHODS

Siliconized glassware was used throughout, and blood was collected by the two-syringe method. The anticoagulant solution used was 3.8 per cent (w/v) sodium citrate in a ratio of 1 part to 9 parts of blood.

Normal blood was obtained from blood bank donors and laboratory personnel.

Human serum was obtained by collecting the blood in tubes containing a few glass beads. The material was shaken and then kept in an incubator at 37° C. for one hour. A large batch of serum was prepared, tested for its activity of stable factors, divided in small aliquots, and stored at −20° C. The serum prothrombin time was also determined by the method of Sussman and associates.[4] The serum was used only if this test gave values above 50 seconds, demonstrating virtual absence of prothrombin. The activity of this material did not decrease in storage. It was used in the test system in a dilution of 1 part to 9 parts of 0.9 Gm. per cent of NaCl.

Plasma as source of AHF and factor V was collected from a normal donor just before the performance of each test. This material was also used as substrate. A donor was classified as "normal" following the criteria described under Special Procedures and Results, paragraph c. Sodium citrate 3.8 per cent was again the anticoagulant solution. The blood was centrifuged at 3,000 r.p.m. for 20 minutes, and the plasma was collected avoiding the resuspension of the buffy coat. The use of a Propipette* was found to be advantageous for this purpose. This plasma is platelet-poor and for practical purposes can be considered platelet-free.

PTC, stable factors, and prothrombin were adsorbed from this plasma adding $Al(OH)_3$ (prepared with the method of Bertho and Grassman[5]) in the proportion of 1 per cent (v/v). The mixture was kept in an incubator at 37° C. for from 3 to 15 minutes, with occasional shaking, and then centrifuged at 3,000 r.p.m. for 15 minutes. The prothrombin time of the supernatant was determined by the method of Quick[6] and, if it was greater than 60 seconds, the adsorption was considered effective, and the material was used in the test diluted in the proportion of 2 parts into 4 of 0.9 Gm. per cent of NaCl.

The procedures described above, which were employed to obtain normal plasma and serum, were also followed in the preparation of these materials from subjects under study.

If platelet activity was to be determined, the plasma was first centrifuged at 1,500 r.p.m. for 10 minutes, removed from the bulk of R.B.C. and W.B.C., and then centrifuged at 3,000 r.p.m. for 20 minutes to obtain sedimentation of platelets.

*Instrumentation Associates, New York, N. Y.

Brain extract with platelet-like activity was prepared by the following method: 600 Gm. of beef brain was cleaned of membranes and large vessels, minced with a knife, and washed twice with 600 ml. of saline solution each time. The drained material was then covered with 1,200 ml. of acetone containing 18 mg. of sodium citrate per 100 ml. The tissue was ground in a mortar. The acetone was poured off, and another liter of fresh acetone was added to the material while the mixture was kept in agitation with a glass rod. After 15 minutes, most of the acetone was again poured off, and the solid material transferred to a blender and stirred for 15 seconds. Washing with one liter of acetone in a mortar was repeated twice, then the brain material was separated on a suction filter. The dry substance was washed 5 times with 200 ml. of acetone, using the suction filter after each washing to separate the "cake." Following the final washing, the material was kept under suction overnight. A yield of 90 Gm. was obtained and suspended in 900 ml. of chloroform. The suspension was shaken for 20 hours in an automatic shaker, filtered, the solid part discarded, and the chloroform evaporated by means of suction at room temperature. The evaporation was continued for 10 days. A thick layer of sticky substance was left at the bottom of the Erlenmeyer flask; this was scraped off with a glass rod after adding about 200 ml. of NaCl solution 0.9 Gm. per cent. This material was transferred to a blender and stirred for 3 minutes, then filtered through 4 layers of gauze into a graduated cylinder. This method is basically that of Bell and Alton[7] as outlined by Therriault and Jensen.[8] The end product is an emulsion, the phases of which cannot be separated by centrifugation at 3,000 r.p.m. for 30 minutes even if diluted with distilled water 1:5 or more. The concentration of the brain material was ascertained by drying a known volume of suspension in a desiccator and weighing the residue. The suspension was then diluted with saline so as to contain 100 mg. per milliliter. The potency of this brain extract was tested by the method described under Special Procedures and Results, paragraph a. This material is stable. It was used in the clotting mixture in a concentration of 125 μg per milliliter (1:800 dilution). The brain extract can be stored after mixing with calcium chloride and then be ready to activate the incubation mixture described below.

Platelets were collected by differential centrifugation as described above. In order to obtain quantitatively comparable concentrations of platelets from different subjects, the following method was used. After separating the platelets from 20 ml. of blood, they were washed 3 times with 10 ml. of 0.9 Gm. per cent solution of NaCl in Lusteroid tubes. After the last washing, the material from each sample was suspended in about 1.5 ml. of saline solution and transferred to a McNaught centrifuge tube.* The volume was adjusted to 2 ml. by adding saline. For critical determinations the Bauer-Shenck* centrifuge tube was used. All the tubes were centrifuged in the same run at 3,000 r.p.m. for 15 minutes. The volumes of packed platelets were read and multiplied by 100. The supernatant was poured off, and the volume made up with saline to the figure thus obtained. In this way the concentration of all the platelet specimens was reduced to 1 per cent (v/v). The platelets were resuspended by mixing the sediment with a wire and shaking. From this suspension serial dilutions were prepared, and the potency tested as described under Special Procedures and Results, paragraph b. If the yield of platelets was too low, enough saline was added to the 1 per cent dilution to have a suitable quantity of material and to make a concentration of 0.5 or 0.25 per cent. When plotting the results in such cases, the curve was started at these lower concentrations rather than at 1 per cent.

A M/40 calcium chloride solution was used to activate the clotting systems.

The steps of the technique are essentially as specified in the original description of the thromboplastin generation test,[1] namely, the following elements are mixed in the order specified.

0.3 ml. of $Al(OH)_3$-treated plasma (platelet-free) at 1:5 dilution.
0.3 ml. of serum at 1:10 dilution.
0.3 ml. of brain extract or platelet suspension.
0.3 ml. of M/40 calcium chloride.

*A. H. Thomas Company, Philadelphia, Pa.

This mixture will be referred to as the incubation mixture (IM). The incubation mixture was kept in a water bath at 37° C., and an aliquot of 0.1 ml. of it was added every minute for 6 successive minutes to aliquots of 0.1 ml. of normal plasma together with 0.1 ml. of calcium chloride solution. The time elapsing from the addition of the incubation mixture to the substrate and the appearance of the clot was recorded. If no clot was formed in 50 seconds, a value of 50 was arbitrarily given to that particular tube. In this way, it is possible to start the next determination after one minute, and the entire procedure can be carried on using only two stop watches: a hand stop watch is used to record the incubation time and a pedal stop watch is used for timing of the clotting of the substrate.

When serum or plasma of a subject was to be studied for activity of individual components, the corresponding material was substituted for the normal component in the incubation mixture. This basic technique was at times modified as described under Special Procedures and Results.

The activity of the incubation mixture is proportional to the concentration of its components. If it is desired to compare the activity of the incubation mixture containing patients' material to the activity of that obtained combining normal elements, the following method may be followed. A rack with six 10 by 75 mm. tubes numbered from 2 to 7 is placed in a tray, and enough water is added to cover the lower 2 or 3 cm. of the tubes. Several ice cubes are added to the water. In each tube is placed 0.5 ml. of physiologic salt solution. The normal plasma and serum are transferred to a tube marked 1 together with the 1 per cent concentration of platelet suspension (or brain extract) and calcium as specified above. This mixture is incubated at 37° C. for 4 minutes; then the tube is placed in the rack immersed in the icy water. Without removing the tubes from the water, 0.5 ml. of the incubation mixture is transferred from tube 1 to tube 2 containing 0.5 ml. of saline. The fluid is aspirated and blown out several times. Then 0.5 ml. of material is transferred from tube 2 to tube 3, and so on until tube 7 is reached. Seven tubes containing 0.1 ml. of normal citrated plasma are placed in a water bath. One-tenth milliliter of material from tube 1 is added to the tube containing substrate. Immediately thereafter, 0.1 ml. of calcium chloride is blown into the tube, and the clotting time is recorded. The same procedure is followed for each of the dilutions in the rack. If standard materials are used and the normal donors screened with the method advised under Special Procedures and Results, the curves obtained on different occasions are very similar. The values observed using patients' materials can then be calculated in percentages by the use of this curve.

SPECIAL PROCEDURES AND RESULTS

a. *Checking the Potency of the Brain Extract.*—Serial dilutions of the brain extract were prepared starting from the original suspension containing 100 mg. per milliliter of material.

The thromboplastic activity of each dilution was ascertained with the following procedure:

Three-tenths milliliter of normal, platelet-free, $Al(OH)_3$-treated plasma at 1:5 dilution, 0.3 ml. of normal serum at 1:10 dilution, and 0.3 ml. of a given dilution of brain extract were mixed in this order and activated by calcium (0.3 ml. The mixture was kept for 4 minutes at 37° C. Then, 0.1 ml. of it was transferred to 0.1 ml. of normal substrate together with 0.1 ml. of calcium chloride, and the clotting time was recorded. In one tube, normal saline instead of brain extract was added.

Plotting the individual clotting times in seconds against the various dilutions of brain extract, a curve is obtained which demonstrates the inhibitory action on clotting of the brain extract at high concentrations and its accelerating

activity at lower concentrations (Fig. 1). The concentration at which the maximum activity was detected was chosen to prepare a large batch of material from the emulsion containing 100 mg. per milliliter. This concentration was then used regularly in the subsequent tests. In the particular case described above, the dilution chosen was 1:800 (125 μg per milliliter).

This material can be kept in the refrigerator indefinitely. We have used the same brain extract for 3 years without changes in activity.

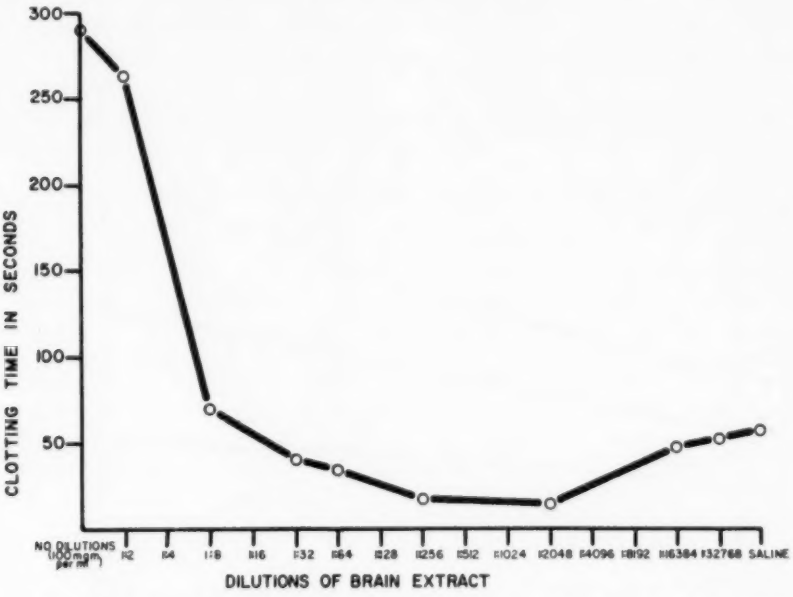


Fig. 1.—Brain extract added (in the amounts noted on the axis) to the incubation mixture composed of: (1) platelet free $Al(OH)_3$-treated normal plasma in 1:5 dilution; (2) normal serum in 1:10 dilution; (3) M/40 calcium chloride. After 4 minutes of incubation, an aliquot of this mixture was added (together with calcium chloride) to normal plasma, and the clotting times were recorded (on the ordinates).

b. *Estimation of Thromboplastic Activity of Platelets.*—Platelets from 45 normal donors were separated, measured volumetrically as described under Materials and Methods, and their concentration adjusted to 1 per cent in saline solution.

The yield from 20 ml. of normal blood after 3 washings is usually about 0.05 ml. However, values ranging between 0.005 and 0.1 ml. have been observed.

From the concentration of 1 per cent serial dilutions up to 1:16 were prepared. The potency of each strength was then tested in the following manner.

Three-tenths milliliter of normal, platelet-free, $Al(OH)_3$-treated plasma at 1:5 dilution, 0.3 ml. of normal serum at 1:10 dilution, 0.3 ml. of platelet suspension, and 0.3 ml. of M/40 calcium chloride were mixed in this order; after incubation for 4 minutes, 0.1 ml. of this mixture was transferred to a normal substrate

together with 0.1 ml. of calcium chloride and the clotting time recorded. Initially, a complete run of 6 tubes at 1-minute intervals was done for each concentration, and the average clotting time calculated as described in the next paragraph. However, this average was not significantly different from the single figure observed at the fourth minute of incubation; therefore, this value only was obtained in order to expedite the performance of the test.

The incubation of the mixtures may be started at intervals of 2 minutes. In this way, there is enough time to determine the activity of each dilution and for the preparation of the successive test, thereby shortening the duration of the entire procedure.

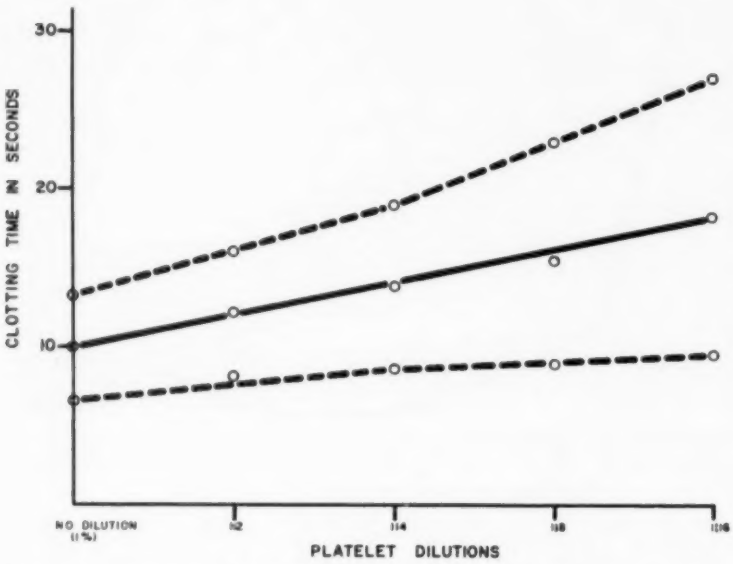


Fig. 2.—Mean values ± 2 $\left(\sigma\sqrt{\frac{n}{n-1}}\right)$ obtained when serial dilutions of platelets were evaluated for their thromboplastic activity with the method described in the text.

Plotting the clotting times against the dilutions, a curve similar to the one represented by a continuous line in Fig. 2 is usually obtained if platelets have normal thromboplastic activity. In this figure, the curve was constructed utilizing the means of 45 tests calculated at each concentration. The mean values and the standard deviations from the mean of the incubation mixture activity at each dilution are given in Table II. The method for calculating the standard deviation of small samples was used $\left(\sigma\sqrt{\frac{n}{n-1}}\right)$. The interrupted lines include the zone between the 2 standard deviations thus obtained.

In the study of a given patient the same technique is used, and the results are reported in graphic form. The curve was considered abnormal if it lay

outside the zone of 2 standard deviations at any point. The procedure customarily employed when the platelet activity is determined by the thromboplastin generation test, namely, reporting the "percentage of thromboplastin formation during 6 minutes" using only one dilution of platelets, was not followed.

TABLE II

	DILUTIONS OF THE PLATELET CONCENTRATES IN SALINE				
	1:1 (1 PER CENT)	1:2	1:4	1:8	1:16
Mean (in seconds)	9.95	11.7	13.3	15.4	18.8
$\sigma \sqrt{\frac{n}{n-1}}$	1.53	2.10	2.72	3.73	4.54

Activity of an incubation mixture prepared by mixing the following: 0.3 ml. of normal $Al(OH)_3$ plasma diluted 1:5; 0.3 ml. of normal serum, diluted 1:10; 0.3 ml. of normal platelets at the specified concentrations; and 0.3 ml. of M/40 calcium chloride. The figures represent the mean values of 45 tests and the standard deviations from each mean.

c. *Plasma and Serum Activity Observed Using Normal Plasma and Sera.*—The thromboplastin generation test was performed according to the classic technique, using material from 100 normal donors. However, the precautions described under Materials and Methods were taken, and brain extract was used throughout at the concentration of 125 μg per milliliter instead of platelets. As stated before, the tests were carried on for a total time of 6 minutes. Usually the incubation mixture reached its maximum activity at the second minute of incubation. However, the clotting times of the substrate at the first and second minutes were disregarded. The clotting times observed at the third, fourth, fifth, and sixth minutes were pooled and the arithmetical mean calculated. We have named this mean value the prothrombin activation index (PAI). The PAI obtained on 100 subjects averaged 9.81 seconds. The standard deviation from the mean was ± 1.44 seconds. The mode was 9, and the median was 9.1.

On the basis of these results, a donor was accepted as "normal" only if his prothrombin activation index was below the mean plus 1 standard deviation, namely, 11.2 seconds.

The prothrombin activation index was calculated for more than 250 subjects suspected of being affected by a hemorrhagic condition. The results were considered abnormal when the values exceeded the mean plus 2 standard deviations, namely, 12.7 seconds. In each patient, the prothrombin activation index was calculated on the mixture of the patient's serum and plasma (complete PAI), on the patient's plasma only, mixed with normal serum (plasma PAI), and on the patient's serum only, mixed with normal plasma (serum PAI). Platelet studies were not performed routinely. Analysis of the results observed in these patients will be the subject of another paper.

d. *Rectification Test.*—When a defective coagulation factor is to be identified, the activity of the patient's plasma or serum is tested by mixing it with corresponding material of previously studied patients who are considered to have a known defect. If, for example, an elevated plasma prothrombin activation index is detected and AHF deficiency is suspected, the rectifying activity of the patient's plasma is evaluated by mixing it with the plasma from a known hemophilic patient. The specific procedure is described as follows:

The elements listed below are mixed together, and the activity of the incubation mixture is checked on normal substrate.

0.3 ml. of hemophilic, platelet-free, $Al(OH)_3$-treated plasma, diluted 1:5 with normal saline
0.3 ml. of normal serum, 1:10 dilution in normal saline
0.3 ml. of brain extract
0.1 ml. of 0.9 per cent NaCl solution
0.3 ml. of calcium chloride, M/40 solution

Then the test is repeated substituting for the saline solution a dilution 1:20 of the platelet-free $Al(OH)_3$-treated plasma of the patient to be studied. If no rectification is observed, the procedure is repeated at lower dilutions of the patient's plasma (1:10 and 1:5). In Fig. 3, a correction of the defect of a

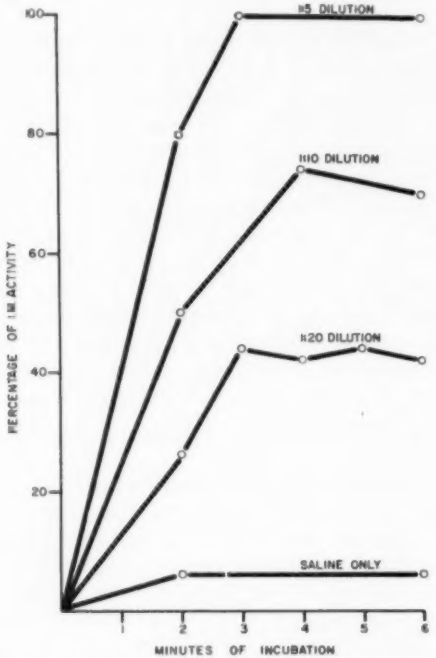


Fig. 3.—Correction of defect of hemophilic plasma by normal plasma. The dilutions of normal plasma indicated in the figure were added to the incubation mixture formed with elements from hemophilic blood.

hemophilic plasma by normal plasma is shown. Fig. 4 shows the lack of correction observed when the plasma from a patient with provisional diagnosis of hemophilia was mixed with the known hemophilic plasma.

The rectifying activity can be expressed in percentage by calculating the activity of the incubation mixture before and after the addition of the unknown

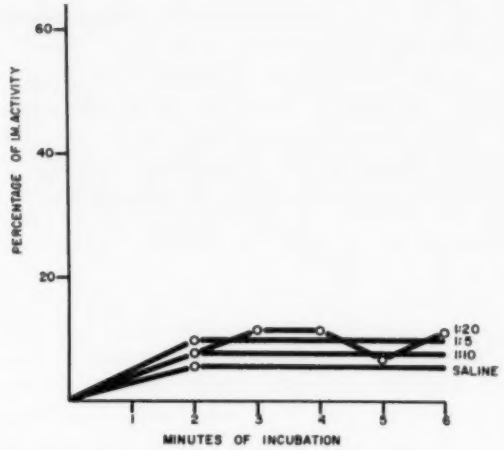


Fig. 4.—Lack of correction of defect of hemophilic plasma by plasma of a suspected hemophilic patient. The dilutions of the suspected hemophilic patient's plasma indicated in the figure were added to the same incubation mixture as in Fig. 3.

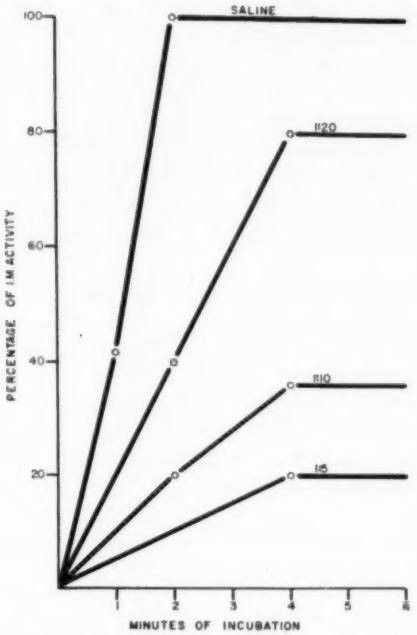


Fig. 5.—Inhibition of activity of normal plasma by a plasma with anticoagulant activity. The dilutions of the abnormal plasma indicated in the figure were added to an incubation mixture formed with elements from normal blood.

plasma by means of the reference curve described under Materials and Methods and in paragraph h of this section.

e. *Detection of Anticoagulants in Circulation.*—A technique analogous to that described above can be used to ascertain if an anticoagulant is present in the circulation. In this case, normal plasma and normal serum are activated by brain extract and calcium, first in the presence of saline solution, then in the presence of plasma or serum of the patient, the proportions and procedure being the same as those described in the preceding paragraph. The test is positive if the addition of the patient's material reduces the activity of the mixture of normal elements.

Again the results of each test can be expressed in one figure by calculating the percentage of prolongation of the prothrombin activation index of the normal mixture before and after the test, as described in the preceding paragraph. In Fig. 5, a test showing anticoagulant activity of plasma is presented.

f. *Estimation of the Severity of AHF or PTC Defect.*—The detection of an abnormally elevated prothrombin activation index not only affords a means of indicating the diagnosis but gives as well an approximate estimation of the severity of the observed defect. If the normal activity of factors other than AHF or PTC which may affect the serum or plasma prothrombin activation index is established, the severity of the defect may be estimated by this index as obtained with the technique described.

If, for example, a plasma PAI is 38, this may mean that there is present an AHF defect or a factor V defect. There are other rarer possibilities, but, for the sake of simplicity, they will not be considered here. If factor V activity is within normal limits, the plasma defect is most probably one of AHF. Factor V activity can be determined by one of the standard methods.

The same reasoning is valid in instances of elevated serum PAI, in which case PTC deficiency or Stuart factor deficiency are the most probable causes for the observed defect.

g. *The "Reversed" Thromboplastin Generation Test.*—An incubation mixture can be prepared with normal plasma, normal serum and normal platelets, activated by CaCl and aliquots of this incubation mixture can be transferred at time intervals to an "unknown" plasma, thus used as substrate. Under these circumstances, the clotting time of the substrate should be a function of certain factors which it contains. In other words, in such an experiment, the same system used for the determination of the Quick time ("prothrombin time")[6] is employed except that brain thromboplastin is substituted by the activated incubation mixture.

To ascertain which elements are indispensable in the substrate, the following experiments were performed.

1. A series of 6 tubes containing 0.1 ml. of purified fibrinogen was prepared. A solution containing 250 units of partially purified prothrombin* per milliliter was prepared and diluted 30 times with saline. Five-tenths milliliter of this solution was placed in a tube. In another tube was placed an aliquot of the acacia-imidazole-calcium (AIC) solution used

*Kindly supplied by Ortho Research Foundation, Raritan, N. J.

in the two-stage prothrombin time test.[9] The proportions of the various elements were, however, acacia 2 parts, imidazole buffer 1 part, calcium chloride 4 parts (instead of 2 parts), and normal saline 2 parts (instead of 4 parts). In a third tube, normal plasma, normal serum, brain extract, and calcium chloride solution were mixed together as described in the preceding paragraphs. At the third minute of incubation 0.5 of the incubation mixture was transferred, together with 0.5 of the AIC solution, to the tube containing the partially purified prothrombin. After 30 seconds, 0.10 ml. of this material was transferred to one tube containing fibrinogen, and the clotting time was recorded. This procedure was repeated every minute for 6 minutes. The shortest clotting time of the fibrinogen solution was 11.2 seconds and was observed at the fourth minute. In the conversion table used for the calculation of units of thrombin in the two-stage prothrombin time technique, this value corresponds to 1.47 units of thrombin. Since the final dilution of the purified prothrombine in this system was 1:180, the figure of 1.47 has to be multiplied by 180. The resulting figure of 264 represents the *calculated* units of prothrombin per milliliter which are present in the system. This figure is very close to the *actual* 250 units per milliliter employed. This experiment was repeated several times with comparable results.

2. The experiment was repeated exactly as before with the exception that the incubation mixture and the AIC solution were added to saline solution rather than to the purified prothrombin. No thrombic activity could be demonstrated.

3. An aliquot of the incubation mixture at the third minute of incubation was transferred directly to an equal volume of 1 per cent fibrinogen. No clot was observed.

4. Plasma obtained from a previously studied[10] case of congenital factor V deficiency (parahemophilia) was used as substrate. The incubation mixture was prepared using normal elements. The "prothrombin time" of the parahemophilia plasma was constantly above 50 seconds, but when the normal incubation mixture was transferred to it, values within normal limits were obtained, the prothrombin incubation index being 7.5. When the activity of the incubation mixture prepared using this patient's plasma was tested on normal substrate, abnormal results were obtained (prothrombin activation index = 42).

h. *Estimation of the Prothrombin Activation Index in Percentage.*—It may be useful for clinical purposes to report the prothrombin activation index as a "percentage of normal" rather than as an isolated figure. In this case, the reference curve constructed with serial dilutions of the incubation mixture as described under Material and Methods may be used.

This curve may also be used for the estimation in percentage of the AHF or PTC activity.

If the material under study is found to have a value higher than 100 per cent, the incubation mixture should be prepared again with the same components and incubated for 4 minutes. At this time the tube is placed in iced water, dilutions of 1:2 and 1:4 in saline are prepared, and their activities are determined on normal substrate. The percentage value thereby obtained may then be multiplied by the dilution factor.

DISCUSSION

The thromboplastin generation test has proved to be a valuable tool in investigating some aspects of the coagulation mechanism in the clinical laboratory. However, its performance is time consuming, and the results may be affected by the use of materials of widely varying activity, yet classified as "normal." The preparation of platelets and their use as a source of thromboplastic material are largely responsible for the protracted duration and lack of

precision of this method. The substitution of brain extract for platelets has been found useful in reducing the error when, in a given situation, the investigation of plasma factors is indicated and the study of platelet activity is not considered necessary. The preparation of the brain extract is relatively simple. Because this substance is active in extremely small amounts and is highly stable, one batch provides enough material for years of use at a constant concentration.

The optimal concentration of brain extract may be determined (as described in Special Procedures and Results, paragraph a) using essentially the same technique as that to be used in future tests.

When the investigation of the thromboplastic activity of platelets is indicated, the thromboplastin generation test as described by Biggs and Macfarlane is of limited value. The preparation of the platelet suspension as described by these authors allows only an extremely vague estimation of platelet activity. As stated before, the volumetric determination of platelets contained in yields from 20 ml. of blood of normal subjects after 3 washings has been found to vary between 0.005 and 0.1 ml. If, as is recommended,[11, 12] the volume is adjusted to one half or one third that of the original volume of plasma, the concentration of one specimen may be 20 times that of another; yet both specimens are assumed to have the same activity. If patients with mild thrombocytopenia are studied, the difference between one patient's platelet preparation and the normal may be extreme. For these reasons, it is doubtful if many cases of "thromboasthenia" or "thrombopathy" diagnosed by this technique can be accepted.

Uniformity of concentration of platelet suspensions obtained from different donors is attempted by a few workers by enumeration and appropriate dilutions. This method, however, does not take into consideration (1) the often pronounced variations in size of platelets, (2) disruption of the elements which is appreciable after 3 washings and leads to the presence of particles of thromboplastic material that are either disregarded in the count or considered erroneously as single platelets, (3) clumping of platelets which makes the count unreliable, and (4) the statistical and technical errors involved in cell counting.

With the technique described above, a 1 per cent suspension is prepared in the same centrifuge tube used for the volumetric determinations, thereby eliminating most of the errors. The 1 per cent strength was chosen because it was found to be close to the minimum quantity of platelets which, when mixed with normal plasma, normal serum, and calcium chloride solution, enable an incubation mixture to cause clotting of the substrate in a time comparable to that obtained when brain extract (125 μg per milliliter) is used. The activity of platelets at this concentration from different normal donors varies within narrow limits. Several dilutions are tested in the case of each patient, should a defect appear only when a small quantity of thromboplastic material is used. The results are plotted as a curve, the reproducibility of which, when normal subjects are studied, is remarkable.

The reliability of the thromboplastin generation test is also affected by the use of different subjects as sources of "normal" plasmas and sera. Since the factors contributed by serum are fairly stable, a large batch of this material was prepared at one time from a single donor, divided into small aliquots, and kept

at –20° C. until it was to be used. The plasma as a source of factor V and AHF must, however, be collected just before the performance of the test; it is, therefore, necessary to define what can be accepted as "normal." Apparent freedom from disease is not a reliable criterion. The activity of plasma is affected by many uncontrollable factors, including aspirin, moderate consumption of alcohol, treatment with antibiotics, and unrecognized liver function impairment of minor degree. Only by statistical evaluation of samples from a large number of donors can the limits of "normal" be established. In donors, free from detectable disease and not taking any medication, the incubation mixture reaches its full activity within 2 minutes of incubation and maintains it for at least 6 additional minutes. The average activity of the reaction mixture may be expressed by one figure alone (the PAI). The interpretation of this figure is related to the meaning of the thromboplastin generation test itself. To what the activity of the incubation mixture is due is still a matter of dispute. "Thromboplastin" is the name most commonly given to the crude tissue extracts which shorten the recalcification time of a plasma which contains factor V, stable factors, prothrombin, and fibrinogen in normal amounts. If it is desired to use the same term "thromboplastin" to indicate a product of reactions occurring physiologically during the process of coagulation, it seems more appropriate to reserve the name for the hypothetical substance which is formed by the interaction of platelets, AHF, and the other factors, the activity of which may be replaced by tissue extracts. The components of the incubation mixture, however, do not react to form a physiologic equivalent of the tissue thromboplastin, since the activity of the incubation mixture is decreased if factor V and Stuart factor are defective. Also, if a factor V deficient plasma is used as substrate and an incubation mixture formed by normal elements is transferred to it, this plasma will behave as a normal plasma, while with tissue extracts in place of the incubation mixture, abnormal results are obtained (see Special Procedures and Results, paragraph g). To distinguish this "physiologic thromboplastin" from the end product of the reaction between platelets, Hageman factor,[13] AHF, PTC, factor V, and Stuart factor, the terms of "prothrombin conversion complex" or "prothrombinase"[11, 12] have been used (see Table I). Whether these terms designate a real chemical entity or are merely a useful convention when referring to a reproducible phenomenon remains for future investigation. "Prothrombinase" may be nothing else but the "serum Aeglobulin" of Seegers.[14]

The incubation mixture prepared with normal elements does not clot pure fibrinogen as demonstrated in paragraph g. Traces of thrombin, however, may be present in it as revealed by the small clot often observed during incubation. The thrombin is probably formed from prothrombin not completely removed from the plasma by the $Al(OH)_3$. Prothrombin also may be present in large amounts in serum. Since serum is used in this test without any chemical treatment, its prothrombin content will be inversely proportional to the activity of the factors involved in the first stage of coagulation.

Whatever the substances responsible for the incubation mixture activity may be, it seems undeniable that this material (incubation mixture) can activate

pure prothrombin as Biggs and Macfarlane[11] have demonstrated and the experiments presented here confirm (Special Procedures and Results, paragraph g, No. 1). It is also clear that the clotting time of the substrate is proportional to the potency of the incubation mixture.

In view of these facts, it seems most probable that the time elapsing between the addition of the incubation mixture to the substrate and the detection of the end point of the reaction is proportional to the amount of thrombin acting on the fibrinogen of the substrate. This is chiefly a product of the prothrombin present in the substrate, activated by the incubation mixture. The prothrombin activation index, therefore, indicates the average amount of thrombin acting on the fibrinogen of the substrate during a given period.

The prothrombin activation mixture varies only within narrow limits when normal materials are used. The standard deviation of the mean of 1.44 seconds demonstrates that the variation of this value in normal subjects are minimal. Although the interpretation of abnormal results will be discussed in another paper, it may be stated now that the expression of values as the prothrombin activation index did not change the diagnostic impression which might be derived from the interpretation of an abnormal curve.

An advantage of this procedure is that it can provide an over-all evaluation of the potency reached by the incubation mixture and then an approximate estimation of the degree of deficiency of the factor or factors responsible for an observed abnormality. The difficulties encountered in the identification of the deficient factor or factors when the thromboplastin generation test is used are, of course, not overcome with the present modifications. Especially precarious is the classification of the serum defects since the thromboplastin generation test yields abnormal results with serum of patients having different deficiencies. In these cases, rectification experiments with plasmas or sera from patients with established diagnoses must be employed.[2] We prefer not to express results as "percentage of normal." The expected deviations from the mean values of the reference curve make such percentage figures highly unreliable. However, such an expression is often employed in coagulation techniques, and it may be considered desirable to give to the clinician a figure describing the approximate degree of an abnormality. In such a case, the "percentage of normal" can be calculated from the curve constructed as described in Materials and Methods and in Special Procedures and Results, paragraph f. The curves are valid as long as the same reagents are used.

The rectification tests and the technique used for the detection of anticoagulants in circulation were found to be valuable diagnostic tools. Expression of the results of the rectification tests in percentages has limited value, due to differences in degrees of deficiencies between various donors of the abnormal material. Such a method can have useful applications if the same donor of the defective plasma or serum is always used. The expression of the anticoagulant activity of a specimen in percentage is more reliable since the inhibition is exercised on normal material. The "reversed thromboplastin

generation test'' can theoretically be used to evaluate the prothrombin concentration of a specimen. Some authors[14] use this procedure to detect the ''antithromboplastic'' activity of a plasma, using it as a substrate to which an incubation mixture formed by normal elements is transferred.

SUMMARY

Certain modifications of the technique generally known as the thromboplastin generation test are presented. They involve:

1. The use of a brain extract as a source of platelet-like activity of constant and measurable potency.
2. A method for the quantitative evaluation of the thromboplastic activity of platelets.
3. The interpretation of the results of a single test on a statistical basis.
4. The application of these modifications to the approximate estimation of antihemophilic factor and plasma thromboplastin component deficiency.

The interpretation of this test is discussed.

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A CAPILLARY TUBE METHOD FOR THE DETERMINATION OF THE COAGULASE REACTION

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WITH the emergence of increasing numbers of drug-resistant staphylococci and the accompanying clinical complications, the need for a more suitable method for evaluating virulence has been evident. Since the earliest observations of Loeb[1] that cultures of staphylococci coagulate blood plasma, little refinement has been made with the coagulase test. Numerous authors[2-5] have investigated the correlation between virulence and the coagulase reaction. This test has proved to be one of the most useful and meaningful tests of virulence. There are at present in general use two methods for the coagulase determination: (1) the tube method and (2) the slide method. Both of these methods are acceptable and give reproducible results. However, when a great number of determinations are required, both of the two previous methods become cumbersome. In addition, the amount of materials required for the test is considerable. If dehydrated standardized plasma is used for the test, the cost becomes prohibitive in a small laboratory. With this thought in mind, a capillary tube method has been devised which produces the same results as either the tube or slide test and can be performed for a small fraction of the previous cost.

MATERIALS AND METHODS

In all cases, 18-hour trypticase soy broth cultures were used for the test. Previously standardized human plasma obtained from outdated blood from the blood bank was used to conduct the tests. Where plasma is not available, a dehydrated standardized plasma can be used. Coagulation tubes No. 3467* were employed for the coagulation tests. These tubes measure 1 to 1¼ mm. in diameter and are 7 cm. long. These tests are set up in a manner similar to the capillary tube precipitation test. About 1 inch of the capillary tube is first filled with 18-hour broth culture by touching the surface of the medium with the tube. Under this, about 1½ inches of plasma is allowed to enter the tube. Easier accessibility of the plasma is provided by placing about 1 ml. in a single concavity of a VDRL test plate. The capillary tubes containing the broth culture are then placed in contact with the edge of the drop of plasma and the plasma is allowed to enter the tube. The tubes are then placed in plasticine clay in a vertical position. The clay can be discarded after use to prevent contamination. Instead of the water bath incubation, as in the tube method, the capillary tubes are placed in a 37° C. incubator for 18 hours. The test is read by observing the formation of a clot at the interface of the broth culture and the plasma in the tube. At the end of a 3-hour incubation period, a clot about 2 mm. in length forms in the strongly

From the Staphylococcus Reference Laboratory, Veterans Administration Hospital, Batavia, N. Y.
Received for publication Aug. 28, 1958.
*Obtained from Arthur H. Thomas Company, Philadelphia, Pa.

positive coagulase reactors. No clot is evident in the negative reactors. In a few cases, incubation should be continued 18 hours to obtain more definite clot formation. All staphylococci studied in this work were submitted to the Staphylococcus Reference Laboratory conducted by the Veterans Administration at Batavia, N. Y.

TABLE I

	CAPILLARY TUBE METHOD		STANDARD TEST TUBE METHOD
Number of tests	300	300	300
Incubation time	3 hr.	18 hr.	3 hr.
Positive reaction	263	257	257
Negative reaction	37	43	43

RESULTS

All capillary tube tests were performed in parallel with the standard tube method[6] in which 0.5 ml. of plasma was mixed with 0.5 ml. of broth culture followed by water bath incubation at 37° C. for 3 hours. A summary of the results obtained with each method is presented in Table I.

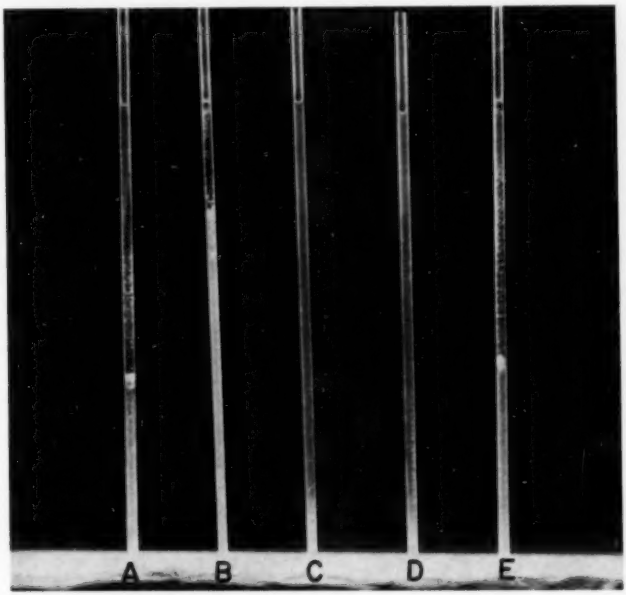

Fig. 1.

In 6 cases there was disagreement with the parallel tests. However, after the 18-hour incubation period had elapsed, the capillary tube results were in agreement with the standard tube test. The clot formation in the capillary tubes becomes more intense after 18 hours, making readings much clearer and easier.

Fig. 1 illustrates the range of the test and the controls required. Tube A is a positive test, Tube B is a weak positive at 3 hours; Tube C is a negative

test; and Tubes D and E are negative and positive controls, respectively. Adequate controls are maintained by storing cultures of known reaction at 4° C.

DISCUSSION

The investigation presented here was begun with the idea of developing a simple, fast, inexpensive test for determining the virulence of staphylococci. In addition, it was thought desirable to have a test which could be performed in a hospital with limited facilities as well as in a large municipal hospital. The fact that this test requires very small amounts of material makes it further adaptable to the smaller laboratories. The cost of materials for the capillary method, where standardized blood bank plasma is employed, is only that of the capillary tubes. These can be purchased already made or can be drawn in the laboratory. There is no glassware to be cleaned and sterilized after the test is conducted, and this saves considerable labor and prevents glassware breakage. Comparisons have been made of the time required for a technician to set up the tube test and the capillary test. The capillary test requires about one-half as much time as the standard tube test. This again results in labor savings. Since greater attention is being focused on the infections due to drug-resistant staphylococci, a test which has a high degree of correlation between coagulase production and virulence is desirable. In the present study, the correlation between coagulase production, phage typeability, and apparent virulence in the human host has been over 92 per cent.[7]

SUMMARY

A capillary tube method for determination of the coagulase reaction of staphylococci is described and a comparison of cost and reliability to the other standard methods is discussed.

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CANDIDA ALBICANS: A STUDY OF MEDIA DESIGNED TO PROMOTE CHLAMYDOSPORE PRODUCTION

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CANDIDA albicans and other yeastlike organisms may be isolated on nutrient agar containing indicators and antibiotics.[1] These media do not indicate that the yeastlike organism is *Candida albicans* and the rapid demonstration of chlamydospores to prove identity is desirable. Taschdjian[2] has described a rice medium containing Tween 80 for the rapid production of chlamydospores and mentions a personal communication from Rosenthal concerning the use of Tween 80 in corn meal agar to improve the production of chlamydospores. Hazen[3] comments that the use of bulk corn meal, used by bakers and not enriched, gives reliable results.

This study has been made to evaluate these media for rapid and abundant production of chlamydospores.

MATERIAL AND METHODS

Eighteen strains of *Candida albicans* isolated from various clinical materials, two Cryptococcus strains and on stock strains each of *Candida albicans, C. parapsilosis, C. guilliermondi, C. pseudotropicalis, C. stellatoidea, C. krusei, C. tropicalis, Rhodotorula mucilaginosa,* and *Cryptococcus neoformans* were used.

Corn Meal Agar.[4]—The materials used were:

Agar	17.5 Gm.
Corn Meal (yellow, bulk)	40 Gm.
Water	1,000 ml.

Place the corn meal in the water and heat to 60° C. for 1 hour. Filter through paper and restore to 1 liter. Add the agar and heat over the flame until the agar dissolves. Dispense into screw-capped tubes, 20 ml. per tube. Sterilize at 120° C. for 20 minutes. The pH was not adjusted. Store in the refrigerator at 10° C. When needed, tubes were melted by placing in boiling water and then poured into sterile petri dishes.

Corn Meal Agar Plus 1 Per Cent Tween 80.—The corn meal agar was prepared as above, but 10 ml. of Tween 80 was added to each liter.

Cream of Rice Agar Plus 1 Per Cent Tween 80.—This was prepared as described by Taschdjian.[2]

Cream of Rice	10 Gm.
Agar	10 Gm.
Tap water	1,000 ml.
Tween 80	10 ml.

To the boiling water sprinkle in Cream of Rice. Boil 30 seconds, let stand 30 seconds, filter through cotton, and add the Tween 80 and the agar. Dispense into tubes, 20 ml. in each, and autoclave.

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Received for publication Aug. 28, 1958.

Nickersons Bacto-Chlamydospore Agar.—The medium was prepared from the dehydrated product purchased from Difco.

The medium in plates were inoculated by the slit method. Permitted to remain at room temperature and observed at 16-, 24-, and 48-hour periods, microscopically. The results are presented in Table I.

TABLE I. COMPARISON OF CORN MEAL AGAR WITH AND WITHOUT TWEEN 80, CREAM OF RICE AGAR WITH TWEEN 80, AND NICKERSONS CHLAMYDOSPORE AGAR FOR THE PRODUCTION OF CHLAMYDOSPORES IN CULTURES OF CANDIDA ALBICANS

STRAIN	CORN MEAL AGAR WITH TWEEN 80			CORN MEAL AGAR			CREAM OF RICE AGAR WITH TWEEN 80			NICKERSONS CHLAMYDOSPORE AGAR		
	16*	24	48	16	24	48	16	24	48	16	24	48
C. albicans 1384	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	O/O	M/C	M/C
C. albicans 1132	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	O/O	O/O	M/C
C. albicans 958	M/O	M/C	M/C	M/O	M/C	M/C	M/O	M/C	M/C	O/O	O/O	O/O
C. albicans 959	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	O/O	O/O	O/O
C. albicans 43	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C
C. albicans 2	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C
C. albicans 7	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C
C. albicans 3813	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	O/O	O/O	M/C
C. albicans 4316	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	O/O	O/O	O/O
C. albicans 3979	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C
C. albicans 4138	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	O/O	O/O	O/O
C. albicans 4061	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/O	M/C	M/C
C. albicans 1121	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	O/O	M/C	M/C
C. albicans 1534	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C
C. albicans 1132	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C
C. albicans 1384	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/O	M/C	M/C
C. albicans 1385	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	O/O	M/C	M/C
C. albicans 1831	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	O/O	M/C	M/C
C. albicans stock	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	M/C	O/O	M/C	M/C
C. parapsilosis	M/O	M/O	M/O	M/O	M/O	M/O	O/O	M/O	M/O	M/O	M/O	M/O
C. guilliermondi	M/O	M/O	M/O	O/O	O/O	M/O	O/O	M/O	M/O	O/O	M/O	M/O
C. pseudotropicalis	O/O	O/O	M/O	O/O	O/O	M/O	O/O	O/O	M/O	O/O	M/O	M/O
C. krusei	M/O	M/O	M/O	M/O	M/O	M/O	M/O	M/O	M/O	O/O	O/O	M/O
C. stellatoidea	M/O	M/O	M/O	M/O	M/O	M/O	M/O	M/O	M/O	O/O	M/O	M/O
C. tropicalis	M/O	M/O	M/O	M/O	M/O	M/O	M/O	M/O	M/O	O/O	O/O	M/O
Rhodotorula mucilaginosa	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O
Cryptococcus neoformans	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O
Cryptococcus species 397	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O
Cryptococcus species 630	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O	O/O

*These numbers indicate hours of incubation.

Key to symbols used in the chart: M/C = mycelium and chlamydospores; M/O = mycelium, no chlamydospores; O/O = no mycelia or chlamydospores.

RESULTS

All strains of *Candida albicans* produced chlamydospores in abundance on corn meal agar with Tween 80 and Cream of Rice agar with Tween 80. Chlamydospores were produced on corn meal agar without Tween 80, but not in abundance. Bacto-Chlamydospore agar was not a reliable medium, and, as mentioned by Taschdjian,[2] the blue color was an impediment when searching for chlamydospores. Further, the Bacto-Chlamydospore agar did not produce the chlamydospores in abundance after 16 hours at room temperature. Candida species and the other yeastlike organisms tested failed to produce chlamydospore on all media tested.

SUMMARY

The use of corn meal agar with 1 per cent Tween 80 or Cream of Rice agar with 1 per cent Tween 80 gives the best results for rapid and abundant chlamydospore production of *Candida albicans*.

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DETECTION OF CERTAIN SEROLOGIC IMBALANCES IN HUMAN SERA BY AGAR COLUMNS

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A REVIEW by Wodehouse[1] indicated the wide variety of problems to which agar diffusion methods have been applied. Darcy[2] using a plate gel diffusion method (Ouchterlony type) showed that the "precipitin spectra" of normal and tumor-bearing rats were different. His later investigations[3] eventually focused upon a fast diffusing glycoprotein of the mucoprotein variety. Increases in the concentration of this substance were believed to be associated with tissue growth.

In the Oudin type of agar diffusion column[4] external reactants (antigen) are layered on top of an appropriate antiserum-agar mixture (internal reactants) in a thin bore glass tube. The disproportional concentrations and symmetries of the antigens cause them to migrate through the antiserum-agar at different rates. During this movement, precipitinogens and precipitins combine to form zones of various widths and densities at one or more levels. As a general rule, the external reactant concentrations govern the zonal diffusion, and the amount of antibody with which the antigen combines is reflected in the zone densities. The Oudin column was used in this and previous studies.

Since the reports of Darcy previously mentioned were not discovered in the foreign literature until this and preliminary studies were completed, the results reported here independently confirm his findings and extend such analyses to the detection of certain serologic imbalances in human sera. Our previous studies,[5, 6] with 100 normal and 40 abnormal human samples indicated that normal sera, when individually diffused into appropriate homologous rabbit antiserum, all showed a similar pattern of precipitin zones after 120 hours' reaction time at 24° C. Abnormal sera showed aberrant diffusion patterns plus an additional zone. This zone is apparently associated with an increased concentration of a normal serum component(s) similar to mucoprotein or a glycoprotein complex.[6] It has also been possible to rely on certain marker zones in the diffusion patterns. These have been established by absorption techniques as being associated with beta and gamma globulins and albumin. The relative mobilities and densities of these zones together with the 6′ zone (Fig. 1) are apparently sensitive criteria for detecting imbalances in human sera.

This study was concerned with the qualitative and quantitative differences between the agar diffusion patterns of 29 assumed normal persons and the

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Received for publication Sept. 12, 1958.
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patterns of 131 persons hospitalized due to various conditions. An assumed normal was a person who stated he felt well, evidenced no obvious external symptoms of a pathologic condition, and was actively engaged in his usual occupation. An abnormal person was one who was hospitalized and under the care of a medical staff.

MATERIALS AND METHODS

Antigens and Antisera.—A pool of assumed normal human sera was used to prepare the rabbit antisera. Six rabbits were each given a 1 ml. subcutaneous presensitizing injection (approximately 11 mg. nitrogen per milliliters). This was followed 3 weeks later by 4 subcutaneous injections of 1 ml. given every other day. The antisera were tested 7 days after the last injection using the Oudin column method. Animals whose sera showed a good pattern and discriminatory possibilities were bled out by cardiac puncture the following day.

Additional selection of appropriate antisera was made by pooling sera. The pool used in this study is designated Anti-Hu.

The abnormal human sera,* used as external reactants, came from persons who had a diagnosed active condition or were convalescent. The samples were from the following disease categories: vascular (22), metabolic (18), psychogenic (2), allergic (3), blood dyscrasia (7), malignant (8), skin (6), gastrointestinal (8), collagen (10), liver (12), infectious (25), and miscellaneous (10).

Agar Columns.—Detailed methods for preparing the Oudin type of reaction column have been published.[4, 7] Since the antiserum-agar mixture must be kept at 46° C. for filling the columns and since previous work[8] indicated that this temperature may elicit changes in the serum, the antiserum and 0.6 per cent agar (1:1) mixture was made in 5 ml. quantities. To minimize dilution errors, however, the filled columns were chosen at random. All reactions were prepared in duplicate and kept at 24° C. for 120 hours.

The external reactants were also overlaid on normal rabbit serum-agar mixtures. Antigens that showed definite precipitation under these conditions were discarded.

Quantitation of Reactions.—Instrumentation and procedures for the direct photometric graphing of agar column reactions by manual[9] and semiautomatic methods [10, 11] are published elsewhere.

In this study a recently developed automatic system involving the Serum Agar Scanner Instrumentation (SASI)[12] was used. With SASI, agar columns can be quantitated for both zonal diffusion and density with high resolution and the patterns spread out in both width and length when the reaction requires.

For a more precise measurement of the diffusion of the faint 6′ zone (Fig. 1), a binocular microscope (20×) fitted with an optical micrometer was used to measure the relative diffusion of the leading edge of the 6′ zone compared with the leading edge of the albumin or zone 6.

C-Reactive Protein (CRP).—Reactions between the clinically abnormal human sera and commercially obtained antisera to C-reactive protein were done in the prescribed clinical way.[13] Since measuring the precipitates to obtain quantitative answers was considered unreliable,[6] reactions were recorded as positive or negative after 2 hours' incubation at 37° C. followed by 46 hours at 4° C.

RESULTS

Results of the agar column analyses were divided into 4 groups: (1) qualitative evidence of a 6′ or glycoprotein zone below the albumin or zone 6 (Fig. 1); (2) quantitative comparison among sera of the diffusion distances of the

*Clinically abnormal sera samples were generously supplied by Lackland Air Force Hospital, USAF, Lackland Air Force Base, Texas.

6′ zone relative to albumin; (3) quantitative comparison of the mean density ratio between zones 4 and 6 of each sample; (4) quantitative comparison of the mean diffusion distance of zones 4 and 6 of each specimen.

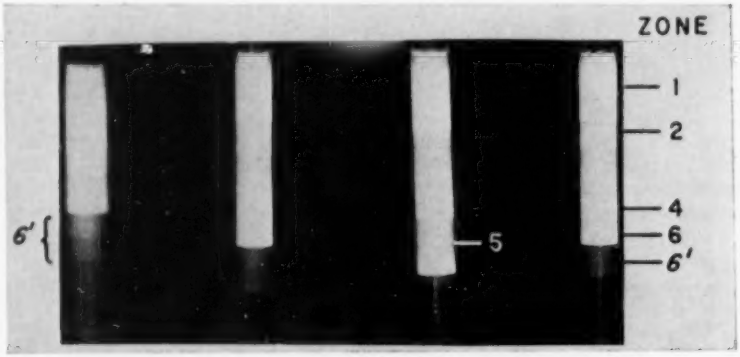

Fig. 1.—Serum agar columns (Oudin type) showing principal precipitin zones when abnormal human serum is diffused into standardized rabbit antiserum (120 hr.). Zone 4 is associated with beta and gamma globulins, zones 5 and 6 with albumin; zone 6′ is associated with glycoprotein. Human sera samples are (left to right): nephrotic syndrome; cirrhosis of liver; myocardial infarction (1½ mo. postaccident); cirrhosis of liver. Normal sera show a pattern similar to the third column but without the 6′ zone.

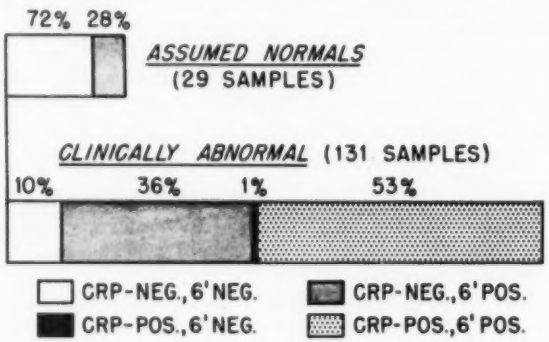

Fig. 2.—Comparison of C-reactive protein test with presence of 6′ zone for human sera samples. Assumed normal samples were taken during winter months when subclinical sinusitis and related infections were prevalent. The 10 per cent in the clinically abnormal group that were CRP negative, 6′ negative were from convalescent patients considered in good condition. Note that 36 per cent of known abnormals showed serologic imbalances but a negative CRP. Additional information can be obtained by relative diffusion and optical density ratios of the precipitin zones shown in Fig. 1.

Fig. 2 shows the incidence of the 6′ zone in all samples. As indicated previously the more rapid diffusion of this zone made it visible below the albumin or zone 6 and is believed to be due to an increased concentration of mucoprotein. In highly abnormal sera, the 6′ zone was often seen as early as after 6 hours' reaction time. Other details concerning this qualitative analysis are included in the legend of Fig. 2.

Table I shows the quantitative data obtained from measurements of zonal diffusion of the 6′ zone, diffusion ratios of zones 6 and 4, and optical density

TABLE I. MEANS AND STANDARD DEVIATIONS* FOR THE QUANTITATIVE ANALYSES OF AGAR COLUMN DIFFUSION PATTERNS RESULTING FROM REACTIONS BETWEEN HUMAN SERA AND ANTIHUMAN RABBIT SERA

	ASSUMED NORMAL				CLINICALLY ABNORMAL			
CATEGORY	NO. OF SAMPLES	ZONAL DIFFUSION 6 to 6′	ZONAL DIFFUSION 6/4 ratio	OPTICAL DENSITY 6/4 RATIO	NO. OF SAMPLES	ZONAL DIFFUSION 6 to 6′	ZONAL DIFFUSION 6/4 ratio	OPTICAL DENSITY 6/4 RATIO
CRP⁻, 6′⁻	21	0	1.40±0.06	1.23±0.05	13	0	1.38±0.05	1.26±0.08
CRP⁺, 6′⁻	0	0	0	0	1	0	1.42	1.26
CRP⁻, 6′⁺	8	1.15±0.40	1.37±0.07	1.23±0.08	47	1.14±0.50	1.36±0.08	1.26±0.08
CRP⁺, 6′⁺	0	0	0	0	70	2.08±0.90	1.33±0.13	1.21±0.08
Total	29				131			

*Significant differences expressed in per cent are explained in the text.

ratios of zones 6 and 4. Of all assumed normal samples with 6′ zones (6′+) 12.5 per cent were beyond the mean ± 1 standard deviation (S. D.) for diffusion of the 6′ zone, whereas 44.0 per cent of all clinically abnormal specimens were beyond this "normal" range. Using the same "normal" range of diffusion for the 6′ zone, 8.7 per cent of the CRP negative (CRP–) and 67.1 per cent of the CRP positive (CRP+) abnormal samples were outside the diffusion range.

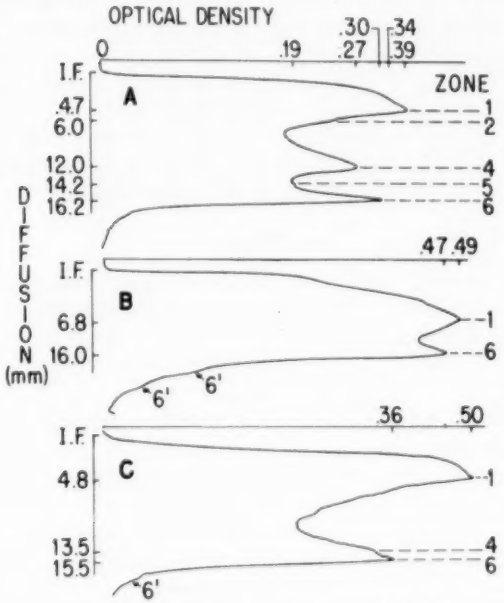

Fig. 3.—Normal and abnormal human-antihuman precipitin zone patterns in agar columns. These graphs were made with the Serum Agar Scanner Instrumentation (SASI).[12] Pattern A, typical normal pattern; pattern B, serum from a nephrotic syndrome; pattern C, serum from suspected malignancy. Reaction time 120 hours at 24° C.

The density and diffusion data for marker zones 4 (beta and gamma globulins) and 6 (albumin) were obtained from the graphic diffusion patterns similar to those already published[6] (see also Fig. 3). Considering only the ratios of the diffusion distances of these zones (D 6/4), 17.2 per cent of all

assumed normal samples were beyond the mean ± 1 S.D., but 29.0 per cent of the clinically abnormal samples exceeded this normal range. The largest percentage (41.4) of abnormal samples to exceed the "normal" range was in the CRP^+, $6'^+$ group. Density ratios of zones 4 and 6 were also computed (O.D. 6/4) for both assumed normal and clinically abnormal samples. In the normal group, 24.1 per cent were outside the mean ± 1 S.D. with the largest percentage (37.5) in the CRP^-, $6'^+$ group. With this "normal" range as a guide, 30.5 per cent of all abnormal samples were outside the limits. The largest percentage (38.5) of abnormal samples that were outside the "normal" O.D. 6/4 mean ± 1 S.D. was in the CRP^-, $6'^-$ group. One agammaglobulinemic patient and one patient who was receiving repeated injections of gamma globulin both showed altered diffusion and/or density ratios for the albumin and globulin zones.

Except for the assumed normal sera used to establish a base-line pattern, all other samples were coded by the physician (R. K.) prior to the laboratory analysis. In this way, neither the source nor the condition of the patient was known to the analysts. A later comparison of the agar column results indicated that in approximately 90 per cent of the samples analysed, the serologic results paralleled the physician's estimate of the stress produced by the illness.

Eleven coded samples not included in the data were received as assumed normals but later serologically classified as abnormal: 91 per cent showed altered density ratios for albumin and globulin, and 73 per cent had the abnormal 6′ zone to a mild degree. In this group, 27 per cent were CRP^+. Further investigation established that these samples were all taken from donors who had been recently immunized with influenza and typhoid antigens.

DISCUSSION

There are indications that latent conditions which do not show clinical evidence of disease or stress may be reflected in altered serum patterns.[6] This might account for the 28 per cent assumed normal samples that were found mildly abnormal as indicated by the appearance of a 6′ zone. These sera were CRP^-.

Later studies with both human and rat sera have emphasized the necessity of standardizing the antiserum pattern by the analysis of a sufficient number of assumed normals. It appears possible from these recent explorations and the reports of Preer and Telfer[14] on diffusion rates relative to antiserum viscosity, that increasing the viscosity of the antiserum might prevent the appearance of the 6′ zone below the albumin zone for a statistically significant number of normal sera. In this manner, laboratory standardization rather than rabbit selection could be utilized. It is obvious, of course, that the production of the correct antibody spectrum is still subject to rabbit variability.

It seems plausible from this and other studies that the antigen fraction manifested as a 6′ zone is a very sensitive but nonspecific indicator of internal stress. It appears to increase in a variety of conditions, some of which were not directly traceable to obvious pathologic symptoms. This substance, believed to

be a mucoprotein, seems to appear in higher concentration before the appearance of a measurable amount of C-reactive protein. Although CRP and mucoprotein have different physical characteristics, they are both glycoproteins and may arise from the same or similar sources within the body. Data reported here points to a lower stress threshold for the increased production of mucoprotein compared to CRP.

The possibility of nonspecific protein interaction and competition for antibody(s) by homologous and heterologous antigen(s) in an agar diffusion column, indicates the necessity for caution in differentiating between normal and abnormal sera solely by means of the diffusion or density ratios of certain zones. Although altered diffusion ratios between albumin and globulin zones of whole sera have been noted to parallel the expected results in agammaglobulinemia and gamma globulin therapy, too few samples from patients with such conditions have been examined to rely only on this type of measurement. The cause of marked altered density ratios (O.D. 6/4) for albumin and globulin zones in sera from recently immunized patients cannot at present be attributed to known alterations in A/G ratios or configurational changes within these fractions.

SUMMARY

Diffusion and density patterns of human-antihuman precipitin reactions in agar columns may be used to detect certain serologic imbalances between normal and abnormal human sera. This method appears more sensitive than the C-reactive protein test and provides some information relative to configurational and/or concentration changes in several serum components.

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THE LABELING OF HUMAN FOODS WITH RADIOACTIVE IRON (Fe^{59})

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INVESTIGATION of the mechanisms and quantitative aspects of the absorption of iron by the human organism has been greatly aided by the introduction of radioactive isotopes of iron. Studies employing inorganic salts of radioiron have provided valuable information concerning the absorption of inorganic iron compounds.[1-10, 15] Valuable as this information is, however, it does not provide information concerning the absorption of iron from foodstuffs in which iron is present as a biologic constituent. Since foodstuffs are normally the primary physiologic source of iron for man, it is evident that quantitative evaluation of this aspect of iron absorption is of prime importance. Balance studies employing chemical analyses have been utilized to obtain such data.[11-14] In addition, the absorption of food iron has been measured after feeding foodstuffs that had been labeled biologically with radioactive iron.[15-18]

We have incorporated radioactive iron (Fe^{59}) of high specific activity into chicken eggs, beet greens, radish greens, swiss chard, algae, yeast, and rabbit liver.[19] The radioactivity of these labeled foods was sufficiently great to permit their use in the evaluation of iron absorption in human subjects.

It is the purpose of this report to describe the methods by which radioactive iron may be incorporated biologically into eggs and vegetables for iron absorption studies. In the techniques to be described, every attempt was made to allow the biologic system to incorporate the iron under normal physiologic conditions.

I. THE INCORPORATION OF $IRON^{59}$ INTO CHICKEN EGGS

Methods and Materials.—

Radioactive iron (Fe^{59}) was procured as ferric chloride from the Isotopes Branch of the Atomic Energy Commission with a specific activity ranging from 1,506 to 1,830 mc. per gram. This was converted to ferric ammonium citrate, and this compound was injected into the wing vein of a laying hen. The amounts given varied between 25 to 40 μc. No adverse reactions occurred after injection of the iron, and the injected hens continued to lay eggs in a normal fashion.

The radioactive eggs were processed in various ways for study. Various components (shell, white, yolk) were separated and their content of radioiron was determined by direct assay in a Geiger-Müller well counter. Radioautographs were made of cross sections of

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Received for publication Sept. 12, 1958.
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hard-boiled eggs to localize the site of the deposited radioactive iron. Chemical studies were carried out to determine the state of combination and affinity of the iron in these compounds.[20] Eggs were hard-boiled or scrambled for patient consumption.

Observations and Discussion.—

1. Time radioactivity relationship: Following the injection of radioactive iron, all the eggs laid contained radioactive iron in varying quantities. The maximum radioactivity usually occurred on the fourth or fifth day after injection, followed by a decrease in radioactivity over a period of 22 to 23 days. At this time, a second increase in radioactive iron content of the eggs appeared, although no second injection was given. This cyclic variation in radioactivity occurred in all 6 hens that we injected and was followed to the start of the fourth cycle in one bird before a second injection of iron was given. The average length of time between succeeding peaks of radioactivity was 26 days. The radioiron activity in eggs layed by the same hen over a period of 100 days is shown in Fig. 1. This curve shows the activity increase immediately after the radioiron injection followed by the second activity increase nearly 24 days later.

Halkett and Peters[20] have shown that this cyclic variation in radioactivity of the eggs is related to the life span of the chicken erythrocyte. This life span has been shown by others[21-24] to range from 20 days with C^{14} to 28 days with P^{32} and N^{15} and to 30 days with Cr^{51}. The radioactive iron incorporated in the chicken erythrocyte at the time of injection is liberated at the end of the life span of the cells and at that time presumably re-enters the metabolic pool, becoming available to the egg-forming organs for incorporation into the developing egg yolks. Thus, the repeating cycles have been explained by the assimilation of radioiron when it becomes available by successive red cell populations, with the liberation of iron at the end of their life span. These findings postulate successful competition for iron between the forming eggs and the erythropoietic system of the hen and raise the interesting question as to which would predominate in iron deficiency.

Although we have not attempted to follow the cyclic variation in radioactivity of the eggs further than the beginning of the fourth cycle (72 days), we have been able to detect radioactive iron in eggs as long as 10 months after the injection of radioiron.

2. Quantitative recovery of radioiron: Two hens were injected on the same day with equal amounts of radioiron. At the end of the second rise in radioactivity in the eggs, or 50 days after injection, the total amount of iron found in the eggs laid by each hen was 53 per cent of the total iron injected.

These observations suggest that the iron content of eggs is dependent upon the iron provided in the chicken's diet. As eggs are a common human food and since iron is one of the important constituents of this food, it is of considerable importance to ensure adequate dietary iron for the chicken.

3. Distribution of iron in the egg: Actual comparison of a cross section of the yolk of a hard-boiled egg with its radioautograph (Fig. 2) has shown that the major portion of the radioiron was found to be in the yolk of the egg.

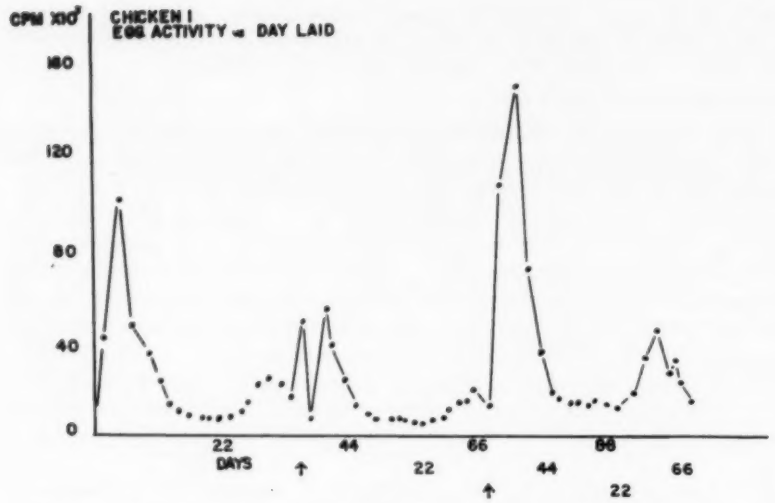

Fig. 1.—The curve represents the individual egg activity on the day laid. This hen was given 3 injections of radioactive iron. The first injection was given at zero time, the other 2 injections are indicated by 2 arrows. Each injection is followed first by an initial egg activity peak and then a second egg activity peak starting 22 days after the injections.

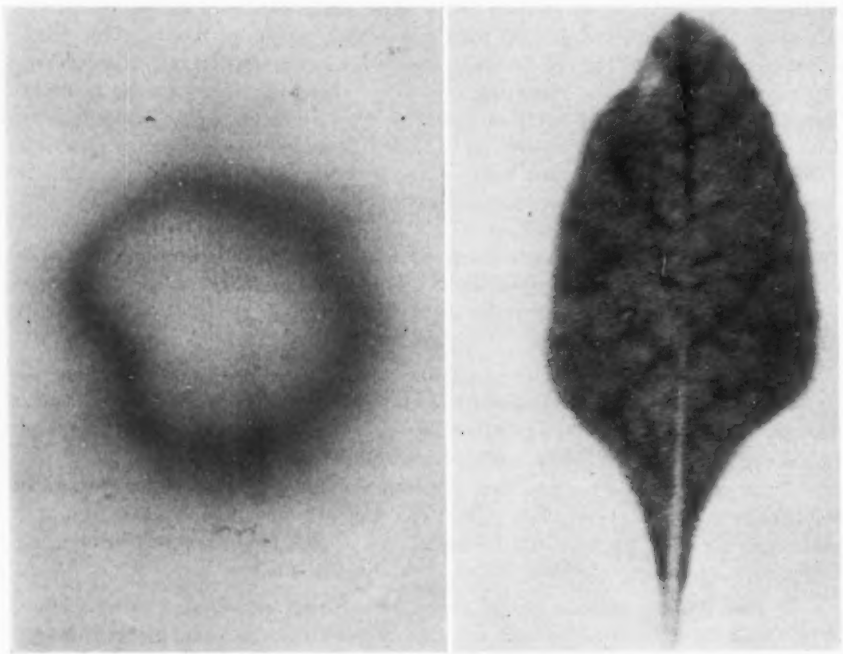

Fig. 2.—Radioautographs of a cross section of the yolk (left) and of a radioactive beet leaf (right).

The quantitative distribution of the radioiron among the different components of hard-boiled eggs was found by direct measurements of the different portions of hard-boiled eggs. The yolk contained 96 per cent radioactive iron, the white had 3 per cent, and 1 per cent was found in the shell and membranes.

II. The Incorporation of Iron^{59} Into Plants

Methods and Materials.—

Employing the technique of hydroponics by which plants are grown in a controlled nutrient solution, and by substituting radioactive iron (Fe^{59}) as ferric chloride for the iron in the nutrient solution, we have succeeded in producing plant foodstuff containing high levels of radioactive iron. The amount of radioiron used was 5 μc per plant. Carrier (non-radioactive) iron, as ferric chloride, was added so that plants have been exposed to iron concentrations varying from 3 to 324 μg. No radiation effects have been noticed over the 30 days of the incorporation experiment.

The plant seeds were germinated in a wooden flat containing vermiculite. On the appearance of the first leaves the shoots were removed from the flat and the vermiculite was washed from their roots with water. The cleaned plants were put into bottles containing 40 ml. of one-third strength Hoagland nutrient solution which was, however, lacking in iron. The plants were held in place in the neck of the bottle by a wad of cotton. The space between the cotton wad and the level of the solution allowed about one-half inch of the roots to be exposed to air (Fig. 3). Algae growth was prevented by using green bottles and placing them in a plaster of Paris mold which kept the amount of light reaching the solution at a minimum.

After 4 days of growth the solution was replaced by a full-strength Hoagland nutrient solution (Table I),[25] plus a known amount of radioiron^{59} as ferric chloride. The pH of the solution remained approximately 5 for the entire growth period. Distilled water was added to replenish the level of solution.

Table I. The Chemical Constituents and Their Concentration Used in the Hydroponic Solution

Modified Hoagland-Arnon Solution	
$NH_4H_2PO_4$	0.0001 M/L.
$Ca(NO_3)_2 \cdot 4H_2O$	0.004 M/L.
KNO_3	0.006 M/L.
$MgSO_4 \cdot 7H_2O$	0.002 M/L.
Micronutrient Supplements (1 ml. of mixture used per liter of nutrient solution)	
H_3BO_3	2.86 Gm./L.
$MnCl_2$	1.81 Gm./L.
$ZnCl_2$	0.105 Gm./L.
$CuCl_2 \cdot 2H_2O$	0.053 Gm./L.
Molybdic acid	0.09 Gm./L.

M = molar solution.

At harvest, which varied from 12 to 30 days after addition of iron and was dependent on the season, the average plant green exposed originally to 3 μg of iron contained close to 1 μc of activity. Fig. 2 is a radioautograph of one leaf. Iron chlorosis was not observed.

As only the greens were used in the feeding experiments, the leaves were cut from the rest of the plant, washed in water, boiled in a small amount of water, homogenized, and then fed to patients or frozen until needed.

Observations and Discussion.—

Temporal relationships of iron incorporation: Beet plants were divided into groups of 10 plants to a group. Each group was identified by the original

amount of iron that the roots of each plant were in contact with. No other iron was added throughout the experiment.

The total radioiron content of the plant and the radioiron content of either the roots or leaves were measured in vivo without apparent damage to the plant,

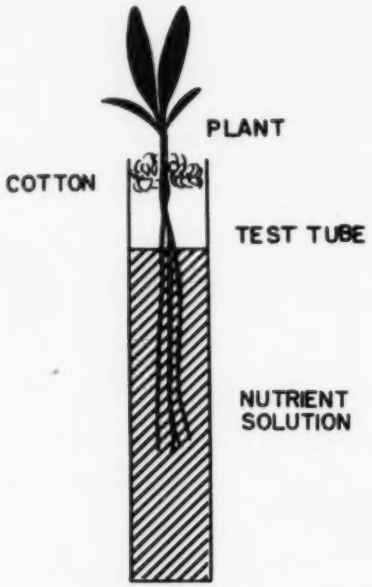

Fig. 3.—Illustration of the technique used to grow a radioactive plant in a hydroponic solution. Details are found in the text.

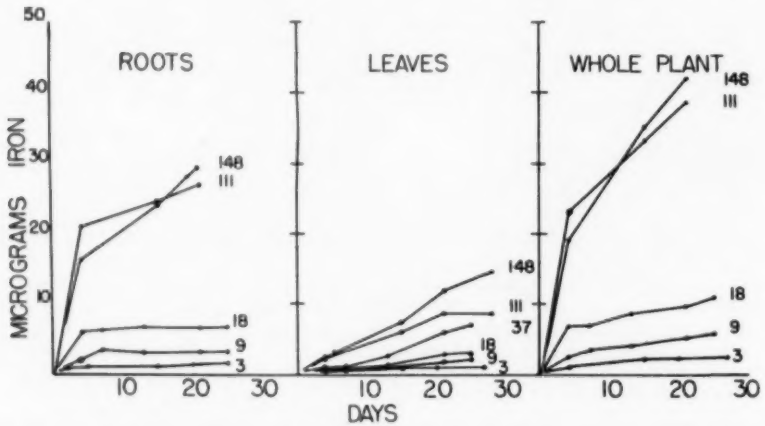

Fig. 4.—The incorporation of iron into growing roots, leaves, and whole beet plants over a period of 28 days. The micrograms of iron in the hydroponic solution at zero time are given by the numbers 3, 9, 18, 111, and 148. These numbers also identify the same plants in each of the 3 chart headings.

using a columnated, lead-shielded Geiger-Müller tube (Sylvania GG306). The average incorporation of iron into the different parts of 10 beet plants is shown in Fig. 4 which is described under each of the following sections.

1. *Roots.* Fig. 4 shows the relationship between time and the micrograms of iron incorporated into the roots for each of 5 groups. Under these experimental conditions, the roots absorbed more iron in proportion to the greater concentration of iron in contact with the roots. Except for the higher concentrations, all groups reached a plateau after the fourth day. This plateau existed throughout the next 22 days.

2. *Leaves.* The relationship between time and the incorporation of iron (in micrograms) into the leaves is shown in Fig. 4. This shows that the greater the concentration of iron to which the roots were exposed, the greater the amount of iron incorporated in the leaves. In contrast to the roots, the leaves did not reach and remain at a plateau except in the group exposed to 3 μg.

3. *Whole plant.* Fig. 4 shows the time-iron incorporation relationship for the whole plant. These results were obtained as the difference between the original iron in solution and the remaining iron in solution. These curves indicate that there was a period of rapid absorption by the plant during the first 4 days when most of the iron was found in the roots. Thereafter, the leaves began to incorporate the iron. These observations serve as a check on the average leaf and root measurements. The greatest per cent of error between the two methods of measurement of total plant iron was 11 per cent.

TABLE II. SHOWN IS THE AMOUNT OF HYDROPONIC IRON THE ROOTS WERE INITIALLY IN CONTACT WITH, THE IRON CONTENT OF THE LEAVES, AND THE PER CENT IRON INCORPORATED AFTER 25 DAYS OF GROWTH*

HYDROPONIC IRON CONCENTRATION (MICROGRAMS)	MICROGRAMS IRON IN LEAVES ON DAY 25	PER CENT IRON INCORPORATED IN LEAVES ON DAY 25
3	0.93	31
9	1.98	22
18	2.9	16
37	6.8	18
111	8.5	8
148	13.5	8

*It illustrates, also, the increase in iron content with increased hydroponic iron concentration and a decrease in efficiency of iron incorporation with increased hydroponic iron concentration.

Per cent of incorporation of iron in the leaves: Table II shows the amount and per cent of iron incorporated in the leaves for each iron concentration originally in contact with the roots. Although a greater quantity of iron was found in plant leaves when the plant was in contact with larger concentrations of iron, the greatest per cent of iron was incorporated at smaller concentrations.

SUMMARY

1. Techniques have been presented for the incorporation of radioactive iron into chicken eggs and vegetable greens. The iron is incorporated into these

foods by normal biologic processes and the labeled foods may be utilized to evaluate iron absorption in human subjects.

2. Radioautographs of eggs and direct measurements of the different components of hard-boiled eggs have shown that almost all of the radioiron is concentrated in the yolk.

3. Measurement of the total activity of individual eggs has shown periodic peaks of radioiron incorporation at approximately 26-day intervals.

4. Radioautographs of vegetable (beet) leaves and direct assay of plant components have shown the effective incorporation of iron into the leaves.

5. Radioactive iron measurements have shown the greater the iron concentration in contact with the roots, the greater the amount of iron absorbed; however, the greatest efficiency of iron absorption was found at the lower concentrations of hydroponic iron.

We wish to thank Drs. Lutz, Fulton, and Harris of the Department of Biology, Boston University, for their cooperation and use of Department facilities during the growth of the radioactive vegetables.

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Errata

In the article by Harold J. Elliott, B.A., and Wendell H. Hall, M.D., Ph.D., entitled "A Comparison of Erythromycin With Oleandomycin in Combination With Other Antibiotics Against Hospital Staphylococci," which appeared in the March issue of the JOURNAL, pages 364-375, the headings of the last column in Tables I and II should read "over 100" instead of "100." The second sentence in paragraph 5 of the Summary should read "Potentiation of bactericidal activity (replica plating method) was frequent with bacitracin and neomycin." In Table VI the name of the antibiotic "Sigmamycin" is misspelled.

In the article entitled "Abnormal Urinary Amino Acid Patterns in Acute Intermittent Porphyria," by Sherman M. Mellinkoff, M.D., Richard M. Halpern, M.D., Marjorie Frankland, A.B., and Margaret Greipel, A.B., which appeared in the March issue of the JOURNAL, pages 358-363, the end of the second line of the legend for Fig. 2 (p. 360) should read: "11, methyl" instead of "1, methyl" and the last line of the same legend should be "(10)" instead of "(4)."

THE DETERMINATION OF SEROTONIN (5-HYDROXYTRYPTAMINE) IN HUMAN BLOOD

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SEROTONIN (5-hydroxytryptamine) is present in the platelets of human blood. The amount of this amine has been estimated by chemical means[1] to be 0.1 to 0.3 γ per milliliter of whole blood. Prior to the present work, and because of the lack of a sufficiently sensitive chemical method, serotonin has been determined by measuring the amount of the amine per platelet, as previously described.[1] A bio-assay method, using the isolated heart of *Venus mercenaria*, has also been used. By this method, Green and associates[2] found the range for the whole blood level of serotonin to be 0.03 to 0.14 γ per milliliter in a small group of neuropsychiatric patients.

In the present procedure, the blood proteins are precipitated with zinc sulfate and sodium hydroxide. One of two methods can be used to purify further and to concentrate the serotonin. Finally, the amount of serotonin is determined by means of its fluorescence.

MATERIALS AND METHODS

Chemicals.—Reagent grade chemicals and solvents were used. All aqueous solutions were made with deionized water.

(1) $ZnSO_4 \cdot 7H_2O$: 10 per cent. (2) NaOH: 1 N. (3) Disodium ethylenediaminetetraacetate (EDTA): for anticoagulation, 1 per cent in normal saline; for analysis, a saturated solution in water. (4) "Salt mixture": 125 Gm. of anhydrous Na_2SO_4 mixed thoroughly with 40 Gm. of $Na_2PO_4 \cdot 12H_2O$. (5) Ethyl acetate. (6) n-Butanol. (7) n-Heptane, prepared by washing commercially available n-heptane with an equal volume of 0.01 N NaOH, then 0.01 N HCl, and finally twice with distilled water. (8) Fifty per cent ethanol. (9) Serotonin creatinine sulfate for standard solutions. From a 100 γ per milliliter stock solution in water kept in the refrigerator, a 1 γ per milliliter solution was prepared each day. (10) Cotton acid succinate (CAS) and columns: prepared as described by McIntire and associates.[3] Into each small glass column, 100 mg. of CAS was packed. The tips of the glass tubes were pointed to permit easy and accurate removal of the final drop of water which had passed through the column. The rate of flow of the organic solvent was regulated by means of a screw clamp and a rubber tube attached to the bottom of each column. The glass tubes were kept in a wooden rack attached to a burette stand so that their height could be adjusted as desired.

All glassware used to hold and to measure the blood before precipitation of the proteins was siliconized. All other glassware, after being washed, was soaked for 2 hours or more in dilute HCl to remove any detergent. This precaution was taken because some detergents were found to cause interference in the fluorometric assay. The glassware was then thoroughly rinsed with tap water, distilled water, deionized water, and finally dried in an oven.

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Received for publication Sept. 15, 1958.

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The Aminco-Bowman spectrophotofluorometer was used for measurement of fluorescence, and for recording the activation and fluorescence spectra.

Procedure.—The blood was drawn using 10 per cent by volume of EDTA (1 per cent solution) as anticoagulant. For each determination, 12 ml. of deionized water was added to 3 ml. of blood in a 30 ml. glass-stoppered shaking tube. The tube was shaken vigorously for one minute in order to lyse the cells. The following reagents were then added in sequence and mixed by shaking for one minute after the addition of each solution: (1) 3.5 ml. of 10 per cent $ZnSO_4$, (2) 2.1 ml. of 1 N NaOH, and (3) 1.5 ml. of EDTA (saturated solution). The contents of the tube were poured into a plastic centrifuge tube and centrifuged in the cold at 14,000 r.p.m. for 30 minutes. To 18 ml. of the clear supernatant solution, in a 75 ml. glass-stoppered shaking tube, was added enough of the "salt mixture" (7 Gm.) to saturate the solution.* The resulting pH of the solution was approximately 12.5. The remainder of the assay was done by one of two methods to be described.

Method 1: To the solution saturated with the "salt mixture" were added 40 ml. of ethyl acetate, and the tube was shaken for 5 minutes. After centrifugation, 38 ml. of the ethyl acetate was run by gravity through a CAS column at the rate of approximately 1 ml. per minute. After all the ethyl acetate had passed through the column, the rubber tube for regulating the flow of solvent was removed, and the CAS was washed by running through the column 3 ml. of 50 per cent ethanol followed by 3 ml. of deionized water. The last drop of water was carefully removed by touching a clean beaker to the pointed tip of the column. A small test tube was placed under the column and the serotonin was eluted from the CAS† with 0.6 ml. of 5 N HCl followed by 0.4 ml. of deionized water. The last drop was carefully removed into the test tube. The fluorescence of the serotonin was then measured in the spectrophotofluorometer at 540 mμ, with the activation wave length set at 300 mμ.

Method 2: To the solution saturated with the salt mixture, in a 50 ml. glass-stoppered shaking tube, was added 20 ml. of n-butanol. The tube was shaken for 3 minutes and centrifuged. The butanol layer increased in volume with water. By means of a pipette, a total of 21.5 ml. of the butanol layer was added to 20 ml. of washed n-heptane, and 0.1 ml. of 2 N HCl in a 50 ml. glass-stoppered shaking tube. After shaking for two minutes, the tube was centrifuged. The heptane-butanol layer was carefully and completely removed by suction. The volume of the acid layer in the tube was 0.9 ml. Exactly 0.7 ml. of this solution was carefully measured and transferred into a small test tube and 0.2 ml. of concentrated (12 N) HCl was added. After mixing, the fluorescence of the serotonin was determined as before.

Duplicate samples were used in all cases. Whole blood alone, whole blood plus serotonin, to measure the recovery of the standard, standard serotonin solutions, and a blank were run through the entire procedure.

Although normal plasma does not contain a measurable amount of serotonin, in experiments designed to study the release of this amine, initial work has shown that serotonin could be determined by the above methods. The protein precipitation step using $ZnSO_4$ and NaOH was eliminated. To 6 or 8 ml. of plasma was added sufficient water or n-saline to increase the volume to 19 ml., and then 1.5 ml. of saturated EDTA solution was added. The salt mixture, which precipitates the plasma proteins, was added as before, and either ethyl acetate or n-butanol was used to extract the serotonin. The remainder of the assay was carried out as described above.

RESULTS

In Table I are shown the actual readings obtained at the fluorescence peak for serotonin. Values are given for whole blood from 4 different patients, for

*The "salt mixture" does not have to be weighed accurately; approximately the same amount can be added to each tube by means of a small measuring cup.

†The CAS can be used repeatedly. After each experiment, it is treated by passing 15 ml. of 0.5 N HCl through the column, followed by 30 ml. of deionized water to remove all traces of the acid.

serotonin standards, and for whole blood to which the serotonin standard had been added. The recovery of the apparent serotonin in whole blood plus that added ranged from 80 to 100 per cent when compared to the total amount expected. For plasma, recovery of added serotonin was always close to 100 per cent.

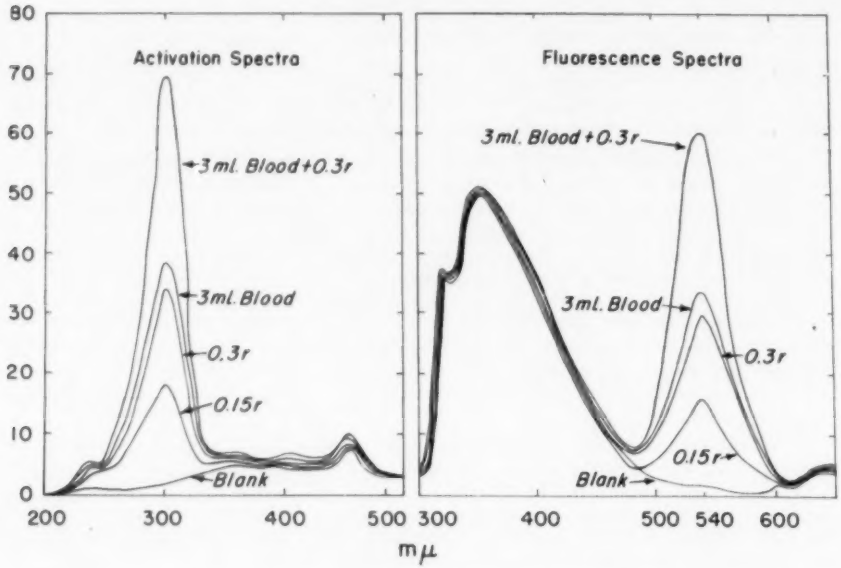

Fig. 1.—*Left*, Activation spectra for serotonin in (1) 3 ml. of human whole blood, (2) 3 ml. of blood plus 0.3 γ of serotonin, (3) 0.3 γ of serotonin, and (4) blank. *Right*, Fluorescence spectra for the same samples.

Fig. 1 shows the activation spectra for the serotonin in whole blood, for the same blood to which a standard serotonin solution had been added, and for the standard solution alone. On the right is shown the corresponding fluorescence spectra for the same samples. The curves for the serotonin of whole blood and for the authentic serotonin standard are similar.

Table II gives the serotonin level per milliliter of whole blood in 21 patients, 10 of whom were normal, with no evident disease. The range of serotonin for these 10 individuals was 0.09 to 0.18 γ per milliliter of whole blood, and the average was 0.13 γ per milliliter. Three of the remaining patients had been taking reserpine (1 mg. per day) for at least one month before blood was drawn for analysis. The extremely low whole blood level was expected since reserpine has been shown to release serotonin from human platelets in vivo.[4]

Blood from one normal patient was assayed every 2 weeks over a 3-month period. The level ranged from 0.10 to 0.12 γ per milliliter suggesting a narrow range for a normal individual over a period of time.

DISCUSSION

The fluorescence characteristics of serotonin[5] led to the development of a specific and sensitive assay method for this amine in tissues, but the procedure was not applicable to blood.[6] The recently published method for serotonin

TABLE I. SPECTROPHOTOFLUOROMETER READINGS FOR SEROTONIN IN HUMAN WHOLE BLOOD, IN THE SAME BLOOD TO WHICH SEROTONIN HAD BEEN ADDED, AND FOR SEROTONIN STANDARDS

PATIENT	SAMPLE	SPECTROPHOTOFLUOROMETER READING		PER CENT RECOVERY* SEROTONIN	γ/ML.† BLOOD
		DUPLICATES	AVERAGE		
A	3 ml. blood plus	61	62	—	0.14
		63			
	0.3 γ serotonin	96	98	80	—
		100			
B	3 ml. blood plus	70	70	—	0.15
		70			
	0.3 γ serotonin	108	109	84	—
		110			
C	3 ml. blood plus	48	50	—	0.10
		52			
	0.3 γ serotonin	100	102	92	—
		104			
D	3 ml. blood plus	85	84	—	0.17
		83			
	0.3 γ serotonin	134	136	95	—
		138			
	0.15 γ serotonin	28	30	—	—
		32			
	0.30 γ serotonin	59	60	—	—
		61			
	Blank	0	0	—	—

*Calculated on the basis of the expected reading, obtained by adding together the individual readings for whole blood and the serotonin standard.

†Corrected for (1) the recovery, and for (2) the 10 per cent dilution by the original anticoagulant solution.

TABLE II. SEROTONIN WHOLE BLOOD LEVELS IN HUMAN BEINGS

DIAGNOSIS	PATIENT NUMBER	SEROTONIN (γ/ML. OF WHOLE BLOOD)
Normal adult	1	0.10
	2	0.09
	3	0.18
	4	0.10
	5	0.12
	6	0.18
	7	0.10
	8	0.09
	9	0.14
	10	0.15
Rheumatic heart disease	11	0.12
	12	0.10
Constrictive pericarditis	13	0.15
Hypertension, essential	14	0.13
	15	0.09
	16	0.14
	17	0.12
Hypertension, congestive heart failure, mild diabetes	18	0.09
Hypertension, reserpine therapy	19	<0.02*
	20	<0.02
	21	<0.01

*The sign < indicates that a reading above the blank was obtained, but no distinct fluorescent curve for serotonin.

and histamine in blood and tissues by Weissbach, Waalkes, and Udenfriend,[7] although adequate for animal species (rabbit, cat, mouse, rat, and chick) where the serotonin level is 1 γ per milliliter or greater, lacked sensitivity in order to measure the small amount of serotonin in human blood. Malignant carcinoid was the only known condition in human beings where this method could be utilized for blood serotonin.[7] The present procedure has a high degree of sensitivity and is adequate for normal human blood. The values obtained agree satisfactorily with those previously published using the platelet isolation technique.[1]

The amount of $ZnSO_4$ was found to be important. If more than that specified was used, a decrease was noted in the final reading for standard solutions; if less was used, a drop was found in the per cent recovery of the serotonin added to blood. The concentrations of $ZnSO_4$ and NaOH finally employed consistently gave an 80 to 100 per cent recovery of serotonin in whole blood.

Initially, the relative amount of serotonin extracted into the organic solvents was found to be low. The possibility that heavy metal ions might be a factor was considered, and the chelating agent, EDTA, was tried. With the concentration of EDTA as given, the final fluorescence readings for serotonin in standard solutions and blood were almost twice the readings for the same samples run without EDTA.

As previously reported,[7] serotonin can be separated from histamine by means of CAS. This ion exchange medium was originally prepared and studied for histamine purification by McIntire and associates.[3] The same precautions in its use, in general, must be followed as for other weak carboxylic acid resins. However, it has one distinct advantage. As an ion exchange medium, it retains the physical characteristics of cotton. In contrast to other resins, there is no necessity for maintaining a fluid level above the CAS. Instead, each liquid can be allowed to run completely through the CAS before the next one is added.

If serotonin and histamine in n-saline solution are extracted into n-butanol and the butanol layer passed through the ion exchange column, histamine is removed quantitatively by the CAS whereas serotonin remains in the butanol. However, if serotonin is extracted in a similar fashion from a water solution, the serotonin will also remain on the CAS. Further investigation showed that whether serotonin was removed from the butanol depended on the presence of chloride ion. Acetate ion appeared to be similar to chloride ion, whereas the other anions, sulfate and phosphate, and the cation, sodium, caused no problem. When chloride ion was present in the original solution, serotonin remained in the butanol; when no chloride ion was present, serotonin was removed from the butanol by the CAS. If organic acetate solvents (ethyl or amyl acetate) were used instead of n-butanol, and, after extraction, passed through the CAS, serotonin was removed quantitatively from the acetates and retained on the CAS regardless of the presence of chloride ion in the original solution.

In order to measure serotonin quantitatively by the CAS procedure, the final volume, after elution, must be the same from each column. Using 50

different CAS columns, 1 ml. of water was run through each column, as in the actual procedure. The final volume in each of the 50 collecting tubes was measured and compared. The greatest difference in volume of water between any 2 tubes was found to be 0.02 ml. This amount of variation would cause an insignificant error when measuring human whole blood serotonin.

Comparing the two means of extraction, Method 1 was found to give better fluorescence curves, less light scattering, and a smaller fluorescent blank than Method 2. On the other hand, Method 2 is easier to do and can be completed quicker. However, when blood was analyzed for serotonin by both procedures the final results differed by 0.02 γ per milliliter of whole blood or less.

Serotonin is the only 5-hydroxyindole compound that has been found to be present in blood.[7] The method of extraction and purification would eliminate most other 5-hydroxyindoles ever found in animal tissues. The range of values for the serotonin in human blood was found to be 0.09 to 0.18 γ per milliliter. A greater sampling of patients might give a wider range. There was no difference in the value for serotonin in blood from the same patient taken before or after meals. In fact, serotonin levels did not change in blood drawn from a patient before and at 30- to 60-minute intervals for 3 hours after the oral ingestion of 20 mg. of serotonin.[8] The only known condition at present in which serotonin blood levels are higher than this normal range is in patients with malignant carcinoid.[7] However, the blood from patients treated with a monamine oxidase inhibitor, e.g., Marsilid, had a higher level for serotonin than the normal range.[8] The fact that the 3 patients receiving reserpine had essentially no serotonin in their peripheral blood lends validity to the method.

SUMMARY

A sensitive fluorometric method for serotonin assay in human whole blood (and plasma) has been described. After precipitation of the blood proteins, serotonin is extracted from a basic solution into organic solvents and is purified and concentrated by means of an ion exchange medium, cotton acid succinate, or by extraction into acid. The values for blood from 21 patients are given and discussed.

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FLUOROMETRIC MEASUREMENT OF RAT PLASMA AND ADRENAL CORTICOSTERONE CONCENTRATION

A Note on Technical Details

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SEVERAL methods for fluorometric measurement of corticosterone have appeared recently in the literature.[1-5] These publications have been concerned mostly with the basic physicochemical considerations necessary to establish these methods and show their degree of specificity and sensitivity, etc. The purpose of this note is to report the details of the *routine* utilization of one of these techniques[4] with the minor modifications introduced in its original description for the measurement of plasma (2.0 and 0.5 ml. samples) and adrenal corticoid concentration in the rat. The technique in the form reported here has been used on several thousands of samples of rat plasma and adrenals over the past year and its physiologic validation has been reported elsewhere.[7] This technique has also been used successfully in this laboratory (W. Deer, to be reported) with plasma of other species (turtles, *Testudo graeca;* fish, *Ictalurus punctatus;* alligators, *Alligator mississipiensis*).

MATERIALS AND METHODS

Reagents.—

Chloroform (A. R., Eastman Kodak) redistilled from Na_2CO_3.

2,2,4-trimethylpentane (isooctane), practical grade.

Sulfuric acid (A. R., Baker) to make 30 N solution (420 ml. concentrated H_2SO_4 up to 500 ml. with glass-distilled water).

Ethyl alcohol, redistilled from 2,4-dinitrophenylhydrazine.

Sodium hydroxide, 0.1 N solution.

Corticosterone-free alcohol, for various stock solutions (see following) in absolute ethanol; keep in refrigerator.

Equipment.—

Fluorometer: Farrand, Model A fluorometer fitted with the filter system described by Sweat[6] for corticosterone, i.e., primary filter, $\lambda = 436$ mμ; Corning filters 3389 + 5113; secondary filter, $\lambda = 530$ to 545 mμ, Kodak wratten gelatin Nos. 16-74.

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This investigation was supported in part by funds provided under Contract AF 41(657)-224 with the USAF School of Aviation Medicine, Randolph Air Force Base, Texas, and Grant No. A-1276(C2) from the United States Public Health Service.

Received for publication Sept. 22, 1958.

*Fellow of the American Philosophical Society.

Extraction tubes: Glass-stoppered centrifuge tubes, heavy-wall Pyrex (Corning No. 98660 or similar): 50 ml. for plasma macromethod and adrenals; 15 ml. for plasma semimicromethod. Conical centrifuge tubes (12 ml.), heavy-wall Pyrex (Corning No. 8120 or similar), and Teflon plastic caps No. 0, for the adrenal glands.

Stopcock silicone grease may be used without interference.

Methods.—

A. *Plasma, 2.0 ml. sample:* 1. Partition and Extraction. An aliquot of 2.0 ml. of plasma is placed in a 50 ml. centrifuge tube, shaken for 15 seconds with 6.0 ml. of isooctane. The isooctane phase is discarded. Five milliliters of distilled water is added followed by 15.0 ml. of chloroform. The tubes are shaken for 30 seconds, and centrifuged for 3 minutes (about 2,000 r.p.m.). The aqueous layer is removed by aspiration.

2. NaOH Washing. One milliliter of NaOH 0.1 N is added, the tubes shaken for 15 seconds and centrifuged for 3 minutes (about 2,000 r.p.m.).

3. Extraction in H_2SO_4. Ten milliliters of the chloroform extract (bottom layer) are transferred into another 50 ml. glass-stoppered centrifuge tube containing 2.0 ml. of H_2SO_4 30 N. Note time here. The tubes are shaken vigorously for 30 seconds and centrifuged at 2,000 r.p.m. for 3 minutes.

4. Fluorometry. About 1.0 ml. of the acid extract (bottom layer) is transferred into fluorometer tubes. Read 30 to 40 minutes from adding H_2SO_4, the fluorometer being set at *80* for 1.5 μg corticosterone standard.

5. Standard. Three-fourths milliliter of a stock solution containing 2.0 μg corticosterone per milliliter in absolute ethanol is placed in a 50 ml. centrifuge tube and carefully evaporated to dryness by a stream of air. Add 2.0 ml. of distilled water and carry through the whole procedure as above along with unknowns.

B. *Plasma, 0.5 ml. sample*:* The various steps are as in Method A with the following changes in tubes, volumes of reagents and standard.

Five-tenths milliliter of plasma is placed in a 15 ml. Pyrex centrifuge tube. The volume is brought to 2.0 ml. with distilled water. Add 4.0 ml. isooctane; shake 15 seconds and discard isooctane layer. Add 2.0 ml. distilled water followed by 5.0 ml. chloroform. Shake for 30 seconds, centrifuge 3 minutes, discard top layer. Add 0.5 ml. NaOH 0.1 N, as above. Transfer 4.0 ml. of the chloroform extract to another 15 ml. centrifuge tube containing 1.2 ml. H_2SO_4 30 N. Extraction and fluorometry are as above, the fluorometer being set at *40* for a standard of 0.25 μg corticosterone.

Standard. One milliliter of a stock solution containing 0.25 μg corticosterone per milliliter in absolute ethanol is placed in a 10 ml. centrifuge tube and evaporated to dryness. Add 2.0 ml. of distilled water and run through the whole procedure along with unknowns.

C. *Rat adrenals:* One adrenal gland, trimmed of adherent tissue and weighted carefully, is placed in a 12 ml. conical centrifuge tube containing 4.0 ml. saline plus 1.0 ml. absolute ethanol and a few grains of sand. The gland is ground with a glass rod. Add 4.0 ml. isooctane, close with a Teflon plastic cap, shake for 30 seconds, centrifuge for 3 minutes, and discard the isooctane layer. Transfer 4.0 ml. of the ethanol-saline extract to a 50 ml. centrifuge tube fitted with a glass stopper.

The procedure is then similar to Method A but the fluorometer is set to *100* for standard.

Standard. The stock solution is the same as in Method A, i.e., 2.0 μg corticosterone-free alcohol per milliliter, in absolute ethanol. One milliliter of this solution is added to a 12 ml. centrifuge tube containing 4.0 ml. distilled water. This standard is then run through the whole procedure along with unknowns.

*The method has been successfully scaled down to 0.2 ml. plasma.

DISCUSSION

Remarks.—Burettes are used to distribute water, isooctane, saline, ethanol, and chloroform. For the transfer of the chloroform the use of a self-retaining device (Propipette, Instrumentation Associates, New York) has been found considerably time saving.

It has been found convenient to run with each group of analyses only one standard but to have a sample of a pool of plasma (kept frozen in 2.0 ml. aliquots) of known concentration as determined previously in duplicate or quadruplicate in a single experiment. The values for the corticosteroid concentration of these samples of a plasma pool do not vary appreciably for many months.[7]

The exact timing for reading the unknowns in the fluorometer[5] has been determined as that time at which the reading of standard (which will drift upward from 25 to 40 minutes) will remain stable. The "pool" then shows the expected value, usually within 0.1 unit of its previous values.

Calculations.—Standard curves are plotted for each of the 3 techniques on linear graph paper. The curves are of the type $y = a + bx$ where a (due to the minimal background fluorescence found within the reagents when a water blank is run through the procedure) is of the order of + 1.0 for A, + 4.0 for B, and + 1.5 for C. The amount of fluorescent steroids in a sample is read from this graph. It is usually expressed as micrograms of corticosterone per 100 ml. of plasma or per gram of adrenal tissue. Basal or resting levels of plasma free corticosteroids are from 6.0 to 15.0 μg per 100 ml. (mean and standard error: 12.2 ± 0.30 for 128 determinations). The method for determining plasma corticoids as utilized here detects easily 0.02 μg of corticosterone with recoveries from water of 105.0 per cent ± 1.2 and from plasma of 100.3 per cent ± 0.4 at concentrations ranging from 0.1 μg per milliliter to 2.0 μg per milliliter (see ref. 7 for a detailed physiologic evaluation of this method).

SUMMARY

Technical details are reported for the routine use of a fluorometric method to measure adrenal and plasma free corticosteroids of the corticosterone type.

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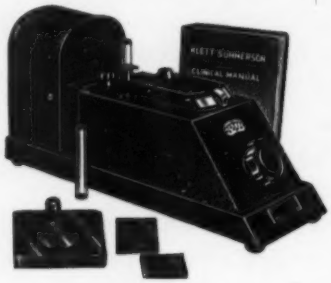

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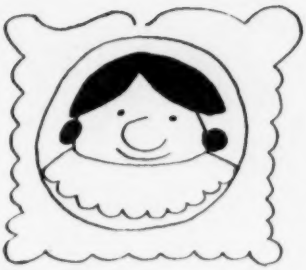

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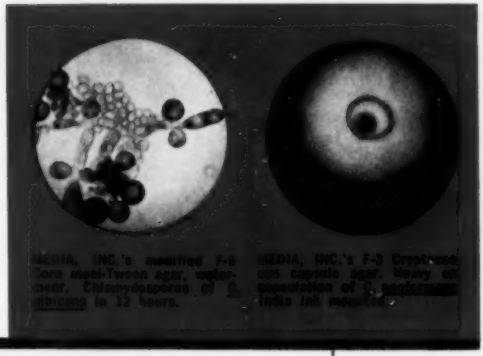

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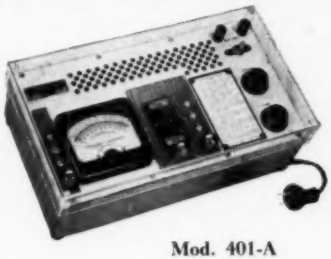

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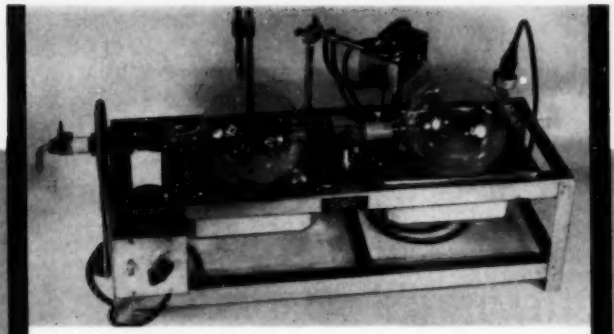

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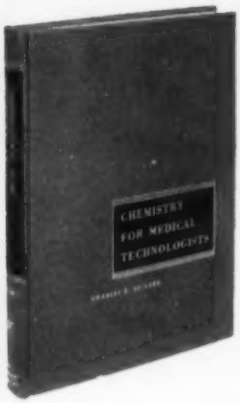

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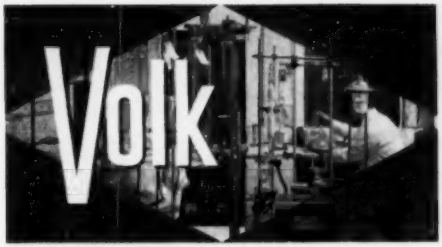

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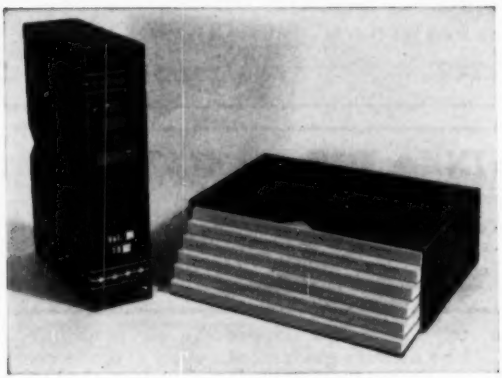

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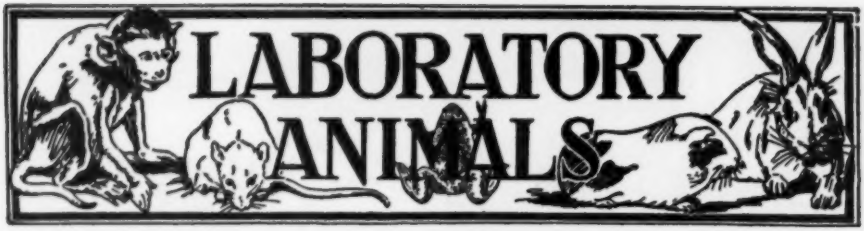

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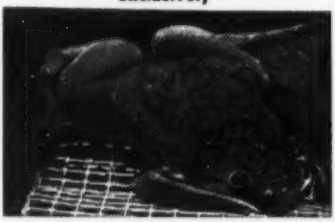

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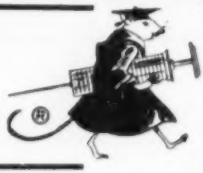

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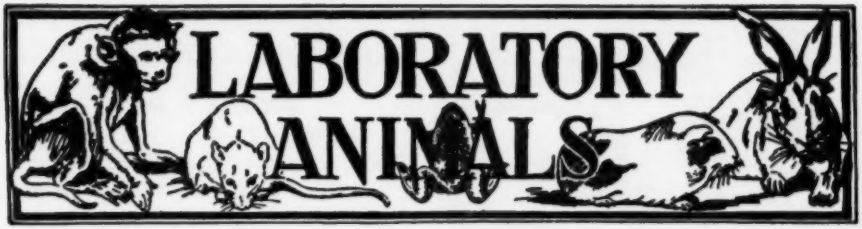

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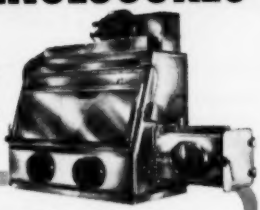

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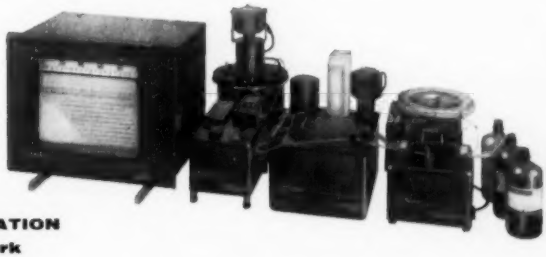

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